

Action, not words

Japan is beginning to recognize that the status and treatment of women researchers must change — but it has yet to take decisive action to address the problem.

The under-representation of women in Japanese science, particularly at its higher levels, is not exactly news. The latest figures on the problem are nonetheless sobering. According to an annual government report on gender equality published in May, fewer than 12% of working scientists in Japan are women — the lowest proportion of any leading industrial nation. Even more strikingly, fewer than 4% of full university science professors are female.

There are some signs, however, that the issue is at last gaining the recognition it deserves. For example, the dismal statistics in the report provoked a barrage of critical coverage in the Japanese press, which might once have been inclined to ignore the issue.

Japanese institutions have started to publicly acknowledge the problem and set targets to redress it. Back in 2000, the Science Council of Japan, which is the interface between Japan's academic societies and the government, said it would raise the number of women on its 210-strong central committee to 10% by 2010. The number, which had hovered around 1% before the announcement, crept past 3% during 2000 and to 6% in 2003.

A cabinet committee on gender equality, meanwhile, set the target of having 30% of all 'leading positions' in society — which should include senior researchers — occupied by women by 2020. A national five-year plan on gender equality, when it is renewed next year, will add the question of women in science to its list of a dozen 'priority objectives'. The Council for Science and Technology Policy, the top science policy body in the government, has also pledged

action. According to one official working on the gender issue, "the little voice of women researchers is starting to be heard".

All of this official activity may start to pull more women into science. But even if that happens, too little is being done to address the set of circumstances that keeps them on the lower rungs of the research ladder, and prevents them from building productive and independent careers.

Junior scientists, for example, are usually dependent on fixed-term grants from research agencies that do not take account of maternity leave. Laboratories in universities and elsewhere make little provision for nursery care. They also lack an accessible and effective body to investigate allegations of discrimination.

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The time is now ripe for the science and education ministry, the universities and the research agencies to put all their fine words into action as they prepare their annual budget requests for submission next month. The Council for Science and Technology Policy will review these requests in the autumn. When it does so, it should consider carefully whether institutions are acting quickly enough to implement Japan's gender-equality objectives. Agencies and institutions that aren't doing so, and prefer to pay lip-service to the issue, need to be made aware that their failure will carry a cost. ■

Two cheers for the G8

World leaders made modest but welcome progress on poverty in Africa and climate change.

Last week's meeting in Scotland of the Group of Eight leading industrialized nations was preceded by the usual game of raising and lowering expectations by pressure groups and the participants, designed to ensure that the summit could afterwards be pronounced a 'failure' or a 'success'. The London bombings of 7 July were timed to undermine the summit, but in fact had the opposite effect. The aftermath of the terrorist attack tempered criticism of the meeting and allowed the G8 leaders to receive credit for at least attempting to tackle the two main items on their host Tony Blair's agenda: Africa and climate change.

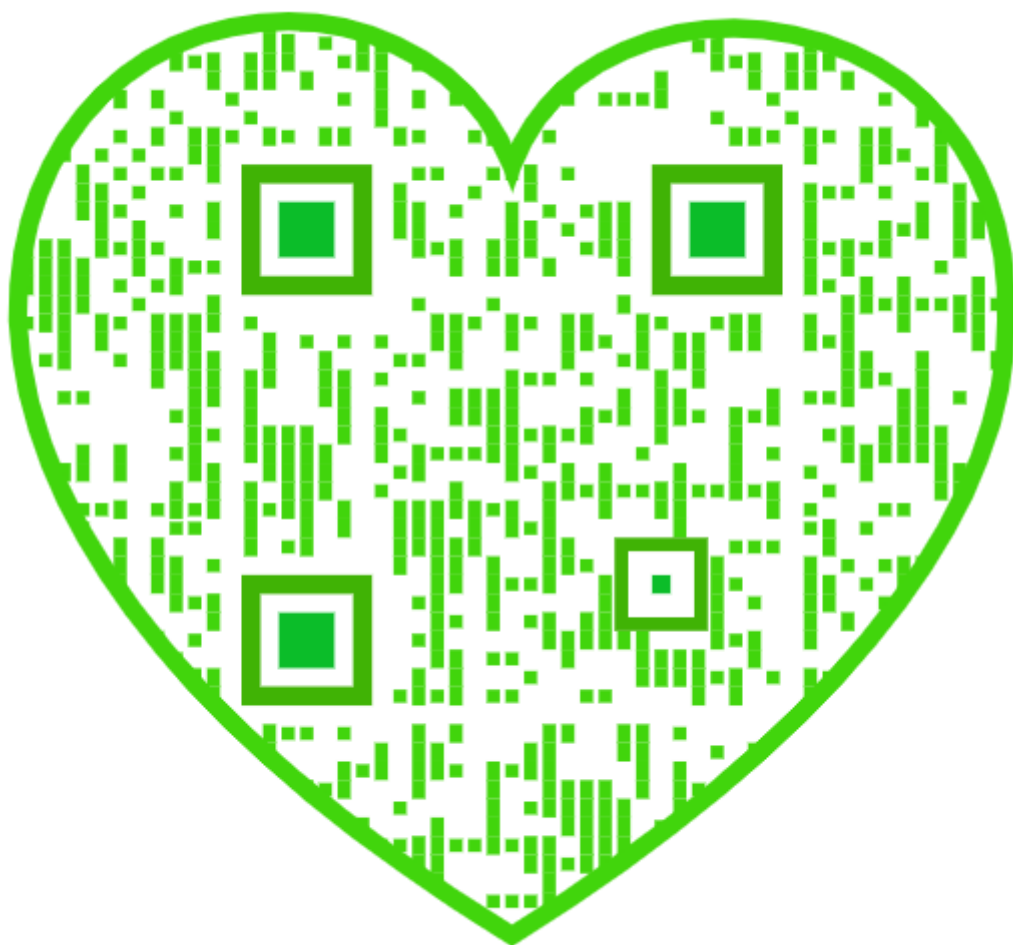
The meeting will not, of course, end African poverty but, on balance, the UK prime minister can be reasonably satisfied with its contribution to that end. No less an authority than Bob Geldof, the Irish rock star who organized a series of concerts and demonstrations to focus public attention on the summit, said afterwards that he gave

the meeting "10 out of 10" for its commitment to increased aid for Africa, and "8 out of 10" for its provisions on debt relief. Few development economists would be as generous as Geldof, but the commitments made at the summit did exceed early expectations.

The G8's recognition that development projects must be African-led is particularly welcome, as is its pledge to tie aid to good governance. In this regard, the Global Fund to Fight AIDS, Tuberculosis and Malaria, which the summit communiqué agreed to refinance, has been leading the way: most of its support goes to countries that show determined and sustained commitment to a project, and to get more money they have to meet agreed milestones.

The communiqué did not say much directly about the role of science and technology in African development. But scientific staff and infrastructure are needed to make policy and tackle disease, develop agriculture and promote an educated workforce. Africa's leaders have recognized this themselves and, through the New Partnership for Africa's Development (NEPAD), are developing plans for science on the continent. It is to be hoped that some of the aid money committed during the meeting will be used to support these plans.

With regard to climate change, the pattern to emerge from the summit is altogether less impressive. Ignoring a plea by the G8



members' scientific academies, the communiqué declined to acknowledge explicitly either the serious threat posed by climate change, or the established facts regarding the central role of fossil-fuel consumption in that threat. It contains some very soft language about the need to "slow and, as the science justifies, stop and then reverse the growth of greenhouse gases".

It is little wonder that environmental groups, and some scientific institutions such as Britain's Royal Society, have roundly denounced the climate communiqué. But even in this verbose document, optimists can unearth some evidence of progress. All of its signatories, including US President George Bush, acknowledge that fossil fuels "contribute in large part to increases in greenhouse gases associated with the warming of our Earth's surface". Some people are interpreting this deliberately vague sentence as evidence that Bush is slowly accepting the science of climate change.

The communiqué also reaffirmed the role of the United Nations Framework Convention on Climate Change (UNFCCC) in responding to climate change. It now falls to the next Conference of the Parties to the UNFCCC in Montreal this autumn to use any political tail wind from the summit to come up with a plan that

looks beyond 2012 and will follow on from the Kyoto Protocol. If that plan contains more flexible, market-led approaches towards promoting cleaner energy supplies and industry than did the Kyoto Protocol, and incorporates meaningful participation by developing countries, there is a chance that the United States will eventually rejoin the process.

By the 2008 G8 meeting in Japan, when the leaders are committed to revisiting the climate-change issue, it is even possible that the United States will be in the throes of a presidential campaign between two main candidates who are each committed to taking the issue seriously.

In the end, the communiqué from the summit meeting may have contained rather fewer concrete steps than Blair hoped for when he set out the agenda. But at least it succeeded in elevating the public profile of two of the most important questions that face humanity. ■

"The communiqué declined to acknowledge the serious threat posed by climate change or the central role of fossil-fuel consumption in that threat."

Diversionsary tactics

A map in a *Nature* supplement is being used to divert debate about science funding in China.

There are good scientists from the island of Taiwan. There are good scientists from mainland China. When *Nature* publishes a paper from Chinese scientists, whether they come from one or the other is not an issue. And whether Taiwan is an independent country — certainly an important and sensitive matter — is not an issue on which *Nature* takes a stand.

It is unfortunate, therefore, that the significance of a map of China in a *Nature* publication has been blown out of proportion and, reportedly, used by some bureaucrats to block several of the supplement's commentaries from wide dissemination.

The map appeared last November in *China Voices II*, a Chinese language supplement devoted to science and science policy in China (and a successor to a similar supplement that was well received a year previously). The map did not show Taiwan. Many Chinese who saw the map admit that they did not even notice the omission, but some immediately claimed that it was a deliberate attempt to portray Taiwan as an independent state.

When *Nature* specifies Taiwan's status, we follow the convention set by the United Nations and describe it either as a 'region' or an 'island'. The map was originally inserted for decorative purposes, and so in recognition of the acute sensitivity in China of the issue of Taiwanese independence, and in view of our neutral position on

"Issues of science funding such as these are universal, controversial, and benefit from open, critically minded discussion."

the question, *Nature* promptly rounded up all the copies of the supplement and destroyed them (a couple of hundred had already been distributed). We then printed several thousand copies of a new version — with no map.

And yet the distribution of the revised supplement continues to be obstructed. At a February session of China's National Congress, officials from the Ministry of Science and Technology were reportedly running about with copies of the original version claiming that *Nature* was in a conspiracy to rip Taiwan from the mainland.

Why have the ministry's bureaucrats reacted in this way? In reality, sources say, they were upset by the content of some of the essays, which were critical of China's science policy. They have sought to use the map controversy to divert attention from the real issues.

The spirit of the supplement was to support the healthy development of science in China, and all articles were written by scientists working in China or intimate with it. Some articles in the supplement are critical. One takes China to task for ploughing too much of its science budget into large, long-term, applied research projects at the expense of smaller-scale, basic science in which, the author argues, individual creativity can flower. Another, more pointed still, cites the lack of peer review in most grant allocation and calls on the Ministry of Science and Technology to surrender control of its science budget to organizations better able to distribute the funds where they are needed most. (In fact, the office of the Minister of Science and Technology turned down an offer to write a commentary for the supplement.)

Issues of science funding such as these are universal, controversial, and benefit from open, critically minded discussion in many countries. The supplement (copies of which can be obtained from chinavoices@naturejpn.com) was intended to foster such debate within China and in the worldwide Chinese community. But such discussion should not be diverted by a false characterization of *Nature's* intentions. ■

RESEARCH HIGHLIGHTS

Island hoppers

PLoS Biol. **3**, 247 (2005)

It was suspected, from archaeological and linguistic data, that Polynesia was colonized by migrants from Taiwan. A team led by Marie Lin, from the Mackay Memorial Hospital in Taiwan, has now provided genetic evidence that supports this view. By analysing the mitochondrial DNA of living populations, the team showed that Polynesians are more closely linked to aboriginal Taiwanese than to the general Chinese (Han) population.

Whether the Polynesian ancestors migrated quickly or spent time on different islands en route to Polynesia is still a matter for debate. Lin's team plans to address the question by studying other communities, including populations in the Philippines and on Borneo.

IMAGE
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DEVELOPMENTAL BIOLOGY

Your beatin' heart

Genes Dev. **19**, 1624–1634 (2005)

The discovery of a zebrafish (*Danio rerio*) mutant has given insight into how hearts beat.

In the *dead beat* mutant, the muscle cells of the fish's heart ventricles fail to contract. Its discoverers, led by Mark Fishman of Harvard Medical School, Massachusetts, and Wolfgang Rottbauer from Germany's University of Heidelberg, traced the fault to a cell-signalling system that controls blood-vessel development.

The disabled gene encodes an enzyme called PLC γ 1, which helps cells to detect a protein involved in vessel growth, known as VEGF. The researchers found that interfering with VEGF signals in rats altered the flow of calcium ions into heart-muscle cells. They suggest that the heart uses such signals to adapt its beat to changes in blood flow.

STEM CELLS

Keeping quiet

J. Cell Biol. **170**, 81–90 (2005)

A signal shown to influence stem-cell proliferation in the ducts of mouse prostates may play a role in the development of prostate cancers.

A team headed by Lynette Wilson of the New York University School of Medicine found that the growth factor TGF- β maintains the quiescent state of stem cells around the ducts near the urethra, while promoting cell proliferation farther away.

Removing androgen, which is an important hormone that regulates prostate growth, reverses the pattern. The researchers suggest that shifts in the balance between these molecules may contribute to proliferative diseases, including cancer.

PLASMA PHYSICS

Bright sparks

Phys. Rev. Lett. **94**, 235001 (2005)

Two researchers from the University of Texas at Austin have identified a promising way to amplify laser power using a plasma of ions and electrons. If the technique holds up in experiments, it could be used to build desktop particle accelerators for medical applications and fundamental physics research.

Serguei Kalmykov and Gennady Shvets calculate that a laser beam travelling through a dense plasma will create a wave that focuses the laser light into a train of sharp pulses — each about 10 to 100 times as intense as the initial beam. A similar technique has been tested with low-power lasers and standard gases, but the duo asserts that using a plasma could push the power of the laser pulses to a thousand trillion watts.

MELANOMA

Skin laid bare

J. Cell Biol. doi:10.1083/jcb.200501067 (2005)

The hormone α -MSH activates the production of the tanning pigment melatonin in skin cells called melanocytes. This pigment shields the skin from cancer-

causing ultraviolet rays. However, stimulation of the hormone's signalling system also upregulates a gene called *Hif1a* that may help cancers to survive, says a team led by Roser Buscá of the French biomedical research institute INSERM in Paris.

The protein encoded by *Hif1a* controls the expression of several genes involved in different aspects of cancer progression, including the synthesis of blood vessels. The researchers show that MITF, a factor involved in α -MSH signalling, promotes transcription of *Hif1a*. This adds to previous results that have linked MITF to skin cancer.

STRUCTURAL BIOLOGY

Sponge path

Nature Immunol. doi:10.1038/ni1224 (2005)

Nature Immunol. doi:10.1038/ni1225 (2005)

Natural killer T cells, key players in the immune systems of animals, are stimulated by lipids bound to CD1d molecules on the surface of cells. CD1d is a unique member of its family of cell-surface binding molecules, because it is found in both humans and other animals, but there has been much debate over what lipids it binds.

Two independent papers, from teams headed by researchers from the Scripps Research Institute in La Jolla, California, and Cancer Research UK, provide a starting point. They describe the crystal structure of mouse and human CD1d in complex with variants of a lipid extracted from a marine sponge. The bound structure may provide clues that help identify CD1d's other natural targets.

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NEURODEGENERATION

The many faces of p53

Neuron **47**, 29–41 (2005)

A study of the pathology of Huntington's disease in human patients and a wide range of animal models has uncovered a protein interaction that may be crucial in producing the disease's symptoms.

The work, led by Akira Sawa of Johns Hopkins University School of Medicine in Baltimore, Maryland, shows that abnormal huntingtin protein binds to the regulatory protein p53. This has the effect of raising p53 levels in cells, causing overexpression of certain mitochondrial genes. Ultimately, this leads to the death of brain cells that is characteristic of the disease.

An overabundance of p53 could also underpin diseases such as Parkinson's, but it may be difficult for therapies to target this interaction because low levels of p53 are linked to cancer.

EVOLUTION

Quagga taggers

Biol. Lett. doi:10.1098/rsbl.2005.0323 (2005)

The quagga, which went extinct at the end of the nineteenth century, was an African animal with distinctive half-zebra, half-horse coloration (pictured). Twenty years ago, it became the first creature to have its DNA studied from beyond the evolutionary grave. Now, an analysis of genetic material from eight museum specimens has been used to determine its pedigree.

Jennifer Leonard at the Smithsonian Institution in Washington and her colleagues estimate that *Equus quagga* diverged from the plains zebra (*Equus burchelli burchelli*) between 120,000 and 290,000 years ago. This is relatively recent and coincides with a point at which Earth's ice sheets were very large, prompting the authors to suggest that climate change may have driven the split.

ASTRONOMY

What's the point?

Preprint astro-ph/0506607 at <http://arxiv.org> (2005)

The orbiting Chandra telescope has completed a huge survey of X-ray point sources in a small area of the southern sky. It turned up 589 new objects, to give a grand total of 915 sources in an area measuring a third of a square degree. Most of the sources are objects known as active galactic nuclei. These are thought to be black holes at the centre of galaxies that emit X-rays as they consume matter. The catalogue will help astronomers to learn about the life cycle and properties of these poorly understood objects.

GENETICS

Mistaken identity

Proc. Natl Acad. Sci. USA

doi:10.1073/pnas.0500398102 (2005)

Identical twins have identical DNA, but their genes gradually diverge as they age, says a study of the young and old.

Manel Esteller of the Spanish National Cancer Centre in Madrid and his colleagues examined chromosomes from 40 twin pairs, aged between 3 and 74. They showed that differences in patterns of gene silencing and activation — caused by the addition of chemical groups to the chromosome — accumulate with age. An illustration of their results (left) maps the differences between twins' sequences on to a chromosome structure — stretches that have not diverged look yellow. Although the trend is not surprising, the twin data uniquely trace environmental influences on gene expression.

JOURNAL CLUB

Michael Marder

The University of Texas at Austin

A statistical physicist finds that taking several paths may be the solution for hard problems.

There are some problems for which finding a solution is like searching for a very small needle in a very large haystack. Take the problem of protein folding. A protein chain can adopt so many different configurations, through complex molecular motions, that modern computational models struggle to predict the shape in which it will settle.

Yet progress in making such predictions is not impossible. Recently, I learned that a community of theoretical chemists and applied mathematicians, somewhat disconnected from the statistical physicists with whom I most commonly correspond, have made great steps forward.

In transition-state theory, the problem of moving from an initial state (such as an unfolded protein) to a final one (fully folded) can be viewed as the problem of moving from one valley to another in a complex mountain range. The task is to find the best path between two valleys, and, in particular, to choose a mountain pass that minimizes the height climbed.

Weinan E, Weiqing Ren and Eric Vanden-Eijnden describe their method for predicting rare events in the *Journal of Physical Chemistry B* (**109**, 6688–6693; 2005). This method uses probability weightings to find not just one path through the mountains, but vast collections of them — some closely related, and others distinct. They apply the technique to predict the rate at which a heated crystal slowly deforms under shear. This is a model problem, but one of such complexity that finding a solution is very impressive.

It almost seems that the communities working to find the likelihood of rare events resemble the solutions they have found, with separate clusters of researchers clambering over nearby but separate mountain passes.

NEWS

Grim but determined — the G8 reaches accord on Africa and climate

Despite the 7 July terror attacks in London, last week's meeting of the Group of Eight industrialized nations remained focused on its two priorities: Africa and climate change. The outcome has left most observers somewhere between disillusion and cautious optimism.

Expectations that the G8 summit would mark a clear turning point in combating climate change or in strengthening science and technology in Africa have clearly not been met (see page 151). But the meeting, held in Gleneagles, Scotland, from 6 to 8 July, created political commitments that may yet yield progress and turned a global spotlight on to Africa.

The communiqué about Africa, signed by world leaders at the summit's close, included measures on debt relief for the poorest countries, funding to combat disease, and a new commitment to double aid to Africa by 2010. But it left key issues dangling on trade and aid, and science was relegated to a back seat.

Hopes that the summit would focus strongly on science and technology were raised by the Commission for Africa, a group of 17 world leaders and heads of UN agencies, whose March report made a strong case for more G8 spending on building science and technology capacity, and training in Africa. The commission suggested donors spend US\$3 billion over the next decade to create centres of excellence, and allot \$500 million every year, for ten years, to reinforcing African universities.

Promises, promises

There are no such specifics in the G8 communiqué. There is, however, a commitment to centres of excellence to help develop skilled professionals, although there is no promise of money nor any clear indication of what such a commitment would mean. This signals an important change, according to Brian Greenwood, director of the Malaria Centre at the London School of Hygiene and Tropical Medicine. "I like the emphasis on investing in people, rather than just on the tools as many other major donors have tended to do," he says.

The summit was more forthcoming on the problem of neglected diseases. It promised to replenish the near-empty coffers of the Global

Fund to Fight AIDS, Tuberculosis and Malaria. "I'm glad. I think this is probably the best vehicle for ensuring sustainable and successful healthcare interventions in Africa," says Richard Tren, director of Africa Fighting Malaria, a pressure group based in South Africa and the United States.

The summit also endorsed universal access to HIV treatment by 2010, adding that it would "support" meeting the \$829-million costs of the Global Polio Eradication Initiative from 2006 to 2008, although it didn't actually commit to giving any money.

Trade-offs

The G8 also agreed, in principle, to incentives for vaccine and drug research on malaria, tuberculosis and other diseases that offer few profits. These include 'advance purchase commitments' in which governments promise to buy, for example, agreed quantities of a malaria vaccine that meets certain performance characteristics. "This is an important development," says Adrian Hill, a malaria-vaccine researcher at the University of Oxford, UK.

Hill adds, however, that he has concerns about how these initiatives would work in practice, and whether they would encourage new companies to enter the field of neglected diseases. He also regrets the lack of direct funding for research on the development of malaria vaccines and says he wanted to see something like the Global HIV Vaccine Enterprise endorsed by 2003's G8 summit. "That led to hundreds of millions of new dollars of HIV vaccine funding from the US National Institutes of Health and the Gates Foundation."

Discussion of easing or scrapping the trade restrictions on Africa, which many feel are the biggest single obstacle to the continent's development, has been postponed until December's World Trade Organization talks in Hong Kong. The summit also failed to clear any debts between individual nations, agreeing only to cancel the debts of the 18 poorest countries to international donors, such as the International Monetary Fund.

In terms of aid, the summit said that growth in spending by G8 and other donors, including commitments made in the run-up to the

summit, is already on track to double aid for Africa to \$50 billion annually by 2010. Britain's proposal for an International Financing Facility, designed to immediately ramp up aid, was diplomatically sidelined as a project that some G8 members believed in, and would continue to consider.

Despite the lack of specific commitments, many observers feel that the G8 got the big picture right. "The best news was probably the high-profile attention given to the problems of Africa and the priority that this now has on the world stage," says Hill. "Hopefully this will not be a one-off. These are huge long-term problems that will require continuing attention at the highest level."

Tren also praises the summit's "strong and repeated messages" that development must be African-led, and that African countries must adopt sound policies for growth and economic development. This echoes calls from African scientists and politicians, published in *Nature*, saying that projects should be run as far as possible by Africans, not donors (see *Nature* 435, 1146–1149; 2005).

Climate of change

The outcome of the climate-change debate was similarly mixed. In a separate communiqué issued on 8 July, the G8 leaders acknowledged that "climate change is a serious and long-term challenge that has the potential to affect every part of the planet".

As expected, the document highlights remaining scientific uncertainties. Nonetheless, the G8 leaders, including US president George W. Bush, agreed that human activities "contribute in large part to increases in greenhouse gases". A list of 38 action points includes the promotion of low-emitting energy systems and vehicles, and a number of market-led approaches aimed at encouraging investment in clean technologies.

The eight nations also promised to start a dialogue with newly emerging economic powers, the G5, about tackling, and adapting to, global warming. The G5 comprises China, India, South Africa, Mexico and Brazil.

Policy analysts say that the most visible success of the meeting was the Bush administration's first public concession that climate change is a real and predominantly man-made threat. However, the noncommittal wording of

BRAIN SCANS BACK UP LORENZO'S OIL
Treatment halts the progress of debilitating genetic disorder.
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Strong suits: the leaders of the Group of Eight nations delivered promises on climate change and Africa, although they provided few financial details.

the communiqué caused widespread disappointment. "The conspicuous failure of the G8 explicitly to mention even the need for targets to reduce emissions of greenhouse gases underlines our concern," says Robert May, president of the Royal Society in London.

Sum it up

The G8 is an informal forum that cannot produce a legally binding treaty. But, says Tom Downing of the Stockholm Environment Institute's Oxford office, the group does have the potential to bring together cities, communities and businesses in the world's richest nations. So he is disappointed that there was no mention in the climate communiqué of how cities and communities might contribute to protection on a regional scale. "The G8 leaders failed to realize this potential," he says. "They basically ignored the policy mandate they were given."

Other climate experts are less harsh. "The substance of the communiqué is better than its rhetoric," says Michael Grubb, an expert on climate change and energy policy at Imperial College London. "I don't think the meeting was a failure. No one ever expected anything

with specific numbers attached to it anyway."

One reassuring signal, he says, is the unambiguous reassertion that the UN Framework Convention on Climate Change is the appropriate arena for discussing a binding climate agreement. The G8 also welcomed Japan's offer to receive a progress report on climate change at the summit it will host in 2008. That's by no means an attempt to sideline the UN, says Grubb. Instead, he believes it indicates that the richest countries, including the United States, have seriously begun to seek a way out of the mess in which climate negotiations have become mired.

What has been lacking in recent years, Grubb says, is a structured discussion that includes both the United States and developing countries. "Now we have a club of 13 core countries, representing the world's major power and population centres, who can attempt to negotiate a feasible climate strategy. This is a quite significant advance."

The meeting may also have provided the first tentative moves towards getting the United States to support the Kyoto Protocol, some analysts believe. The Bush administra-

tion withdrew from the protocol in 2001, saying that it would damage the US economy. But the G8's communiqué and action points include hints that the country may be prepared, in the mid-term, to revise its course, says Axel Michaelowa, head of climate policies

at the Hamburg Institute of International Economics in Germany.

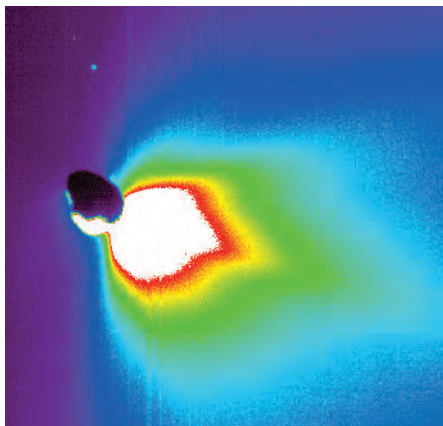
One such signal, he says, is the commitment to adequately fund by the end of 2005 the executive board that oversees the implementation of the Kyoto Protocol's Clean Development Mechanisms. These are measures in which rich countries or industries can buy emission rights if they supply expertise that reduces greenhouse-gas emissions in developing countries. Strengthening technology and market-led approaches in a post-2012 UN climate agreement could get the United States back on board, points out Michaelowa.

"No doubt Bush has left himself a way out," he says. "But the G8 statement includes concessions on the part of the United States that do give hope."

Declan Butler and Quirin Schiermeier

"I like very much the emphasis on investing in people."

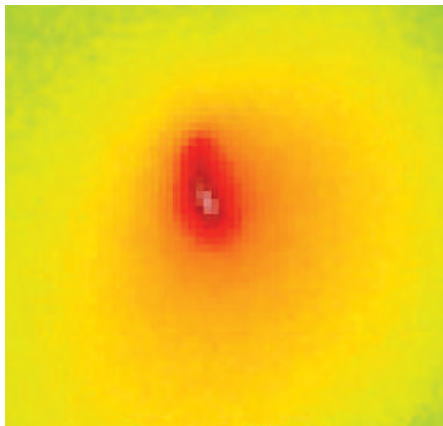
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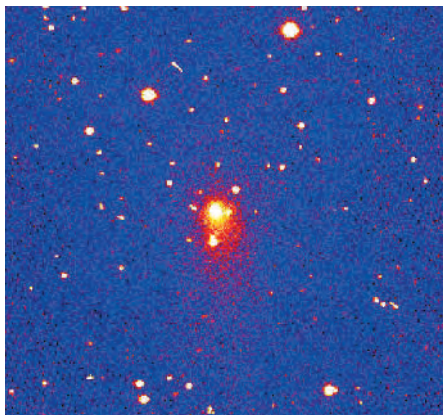
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Deep Impact: sifting through the debris

When Deep Impact's washing-machine-sized probe slammed into comet Tempel 1 on 4 July, teams of astronomers watched using telescopes in space and around the world. *Nature* investigates what the images tell us so far about the comet's composition and history.

The mission's goal was to probe a comet as never before. As the impactor neared the comet's surface at a 25° angle, it took pictures of the landscape beneath that reveal a complex geological history, sculpted by outgassing, melting and natural impacts. Cross-sections that are 20 to 30 metres high display several different layers, confirming that Tempel 1 must have spent time in different orbits. And pictures from the probe's last moments reveal surface features just four metres across, says principal investigator Michael A'Hearn of the University of Maryland, College Park. "That's nearly a factor of ten better than any previous comet mission."

But the aftermath of the collision is throwing up some surprises. The image top left is a false-colour picture taken by the Deep Impact mother ship as it looked back, 50 minutes after the crash. The dusty debris ejected from the impact crater formed a much bigger cloud than expected, and it is still reflecting bright sunlight to scores of telescopes back on Earth.

"That suggests the dust excavated from the comet's surface was extremely fine, more like talcum powder than beach sand," explains A'Hearn. No large chunks of material have been seen, so the surface is unlikely to have an icy crust.

The dust plume was so thick that the mother ship couldn't even see the crater when it looked back. Mission scientists are busy massaging the images to pick out any details, but the diameter of the plume indicates that the crater is between 100 and 300 metres across. Second from top left is an image taken by the Hubble Space Telescope about 64 minutes

after impact. The wider view shows debris extending about 720 kilometres from the nucleus. Rough calculations from the way it has scattered show the comet is much less dense than water ice, and is extremely porous.

As the impactor hit, the Deep Impact team also saw two bright flashes, separated by just milliseconds. This matches simulations conducted by Peter Schultz of Brown University in Providence, Rhode Island, who thinks the first flash came from surface material heated to thousands of degrees. The second, much brighter flash probably came as the molten probe burrowed deeper into the nucleus and hit a layer of more volatile material.

Before the crash, scientists wondered whether the water inside the crater would quickly refreeze to heal the scar created. Instead, the wound has been pumping out material ever since the 372-kilogram, copper-reinforced probe slammed into its target at 10 kilometres per second. Scientists

working on NASA's Swift satellite report that the X-ray glow from the comet is still growing five days after impact. Based on these data, Paul O'Brien, part of the Swift team at the University of Leicester, UK, estimates that several tens of thousands of tonnes of material were released.

Astronomers are particularly interested in what compounds are present in Tempel 1. They are sure that comets played a key role billions of years ago in bringing water from the outskirts of the Solar System to the primitive Earth, which would have been too hot at first to hang on to its own water. But they could have also delivered the carbon and nitrogen that helped to get life established, says Schultz.

Molecular fragments with two and three carbon atoms and nitrogen-containing compounds have already been detected by the UK Schmidt Telescope at the Anglo-Australian Observatory in New South Wales. This supports the idea that the comet's interior may be rich in organics. "But one of the surprises is

"The dust from the comet's surface was extremely fine, more like talcum powder than beach sand."

Top, false-colour image from the Deep Impact mother ship after its probe smashed into comet Tempel 1. Second, image from orbiting Hubble telescope. Third, red-filtered image of dust from a ground-based observatory. Bottom, view from the Rosetta probe, due to meet its own comet in 2014.

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Sparks fly: the Deep Impact craft looks back at the comet's core, capturing the afterglow of the smash.

that we didn't see much gas," adds Andrew Coates of University College London, who is collating data from British telescopes around the world. "Most of it is dusty, grainy material." The third picture on the left is from the European Space Agency's Optical Ground Station in the Canary Islands; it uses a red filter to highlight the dust particles.

Infrared measurements have revealed only weak signatures of water, carbon dioxide and ammonia. "This event did not produce a gusher," says Gary Melnick, principal investigator for NASA's Submillimeter Wave Astronomy Satellite. First estimates suggest the comet produced just 250 kilograms of water per second after the impact, less than in natural

outbursts observed in preceding weeks.

So Tempel 1 was probably not the 'pressure cooker' that some expected. Because the comet passes close to the Sun on each orbit, solar heating could have built up gas pressure inside, releasing a fierce blast when its crust was punctured. Instead, the Sun's heat seems to have baked out volatile materials from the outermost metres, leaving a fine dust of minerals and organics. "Theories about the volatile layers below the surface of short-period comets are going to have to be revised," says Charlie Qi, an astronomer at the Harvard-Smithsonian Center for Astrophysics in Cambridge, Massachusetts.

Working out how comets may have influenced our own origins will take much longer to assess, and will require a mission to bring back samples from a comet. Deep Impact is an important stepping stone, says Joe Veverka of Cornell University in Ithaca, New York. He points out that we now know that comet surfaces are strong enough to land on, yet loose enough to burrow through to retrieve subsurface material, behaving like a layer of fresh snow.

NASA's Stardust craft is expected to bring samples of comet Wild 2's dusty haze back to Earth in January 2006, and the European Space Agency's Rosetta mission is on course to land a probe on comet Churyumov-Gerasimenko in 2014. The bottom picture on the far left shows Rosetta's view of the Deep Impact crash, taken a few minutes after the strike. ■

Mark Peplow

IMAGE
UNAVAILABLE
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A close-up taken by the impactor before it hit the comet shows the surface in unprecedented detail.


**LEAP SECOND TO BE
ADDED TO 2005**

Earth's rotation spins out
of synch with atomic clock.
www.nature.com/news

NASA

K. ROBINSON/PANOS

IMAGE
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Clouded outlook:
researchers'
predictions of
reduced rainfall
in India this
year clash with
government data.

Rival monsoon forecasts banned

NEW DELHI

Researchers at a climate institute in India have voiced concern at what they see as attempts by the government to curb their scientific freedom after they were forced to remove monsoon forecasts from their website. The government says the move is necessary to stop the public being confused by conflicting forecasts. But the scientists are worried that it could prevent researchers in a range of disciplines from communicating their results.

India receives 90% of its rain during the monsoon months of June to September. The economy relies heavily on farming, and so an accurate forecast is critical for sowing seeds at the right time and choosing appropriate crops.

But the monsoon season is notoriously difficult to predict. The country's official forecast comes from the India Meteorological Department (IMD), which is run by the government's Department of Science and Technology (DST). To predict rainfall, it uses a model based on statistics of past monsoons.

In 2002, scientists at the Center for Mathematical Modelling and Computer Simulation (C-MMACS) in Bangalore began forecasting the monsoon with a more sophisticated global-climate model that uses equations to describe Earth's atmosphere. Since 2003, the researchers

have been posting the results on their website, and the Indian media have been reporting them.

Last month, the DST issued a directive prohibiting the publication of any results that differ from its official forecast unless they have been peer-reviewed and cleared by the head of the researchers' agency. The DST will in future collect and disseminate monsoon data produced by research institutes to avoid confusion, DST secretary Valangiman Ramamurthi told *Nature*. "Monsoon forecasting is sensitive for the Indian economy," he says. "It's not a free-for-all."

Prashant Goswami, who heads the monsoon research group at the C-MMACS, says he and his colleagues are upset at the directive. "Posting unpublished results on the web is the accepted norm in science everywhere," he points out. "What upsets us is that we have a monsoon model that seems to work, but we can't make the results public." He is also concerned over the implications for other research fields. "I hope this will be debated by the scientific community," he says.

The DST has not specified the other areas of research to which the directive will apply, although Ramamurthi told *Nature* that guidelines will be issued soon. He says the policy is in the public interest and denies the government

is restricting researchers' freedom. "All we said was that before putting anything on the web, get it cleared by the head of your agency," he asserts.

C-MMACS director Gangan Prathap speculates that the order may have been triggered by the fact that his institute's forecast of rainfall levels 34% below average in June and 12% below average in July is dramatically different from the "normal or above normal" rains predicted by the government.

Madhavan Nair Rajeevan, director of the IMD's National Climate Centre in Pune, admits that the department's monsoon predictions have not improved in the 70 years since they began. "The IMD's model has limitations at district level and its forecasts have been off the mark quite often," Rajeevan told *Nature*. "But it is still the best available for forecasting at an all-India level." He says the clampdown on unvetted forecasts is justified, but adds that his team is looking at switching to a climate model.

In its 27 June bulletin, the IMD admitted that June's rainfall was 35% below average — as forecast by the C-MMACS. Heavy rains in Gujarat over the following three days reduced the deficit to 20%, and the IMD insists that this will be made up in the next few months to make 2005 a normal year, as it predicted. ■

K. S. Jayaraman

ON THE RECORD

“Evolution in the sense of common ancestry might be true, but evolution in the neo-darwinian sense is not.”

Cardinal Schönborn, Roman Catholic archbishop of Vienna, writes in *The New York Times* that the Church's position on evolution has been misunderstood.

“This is about stealing our intellectual property right and left and we're going to have to do something about it.”

Robert Goldberg of the Manhattan Institute for Policy Research criticizes a threat from Brazil to break a US patent on AIDS drug lopinavir.

SCORECARD

Globe trotting
Swiss aviator plans to give wings to renewable energy by flying a solar-powered plane around the world.

Polly math
African grey parrot is first bird to zero in on the concept of 'none' when counting objects — could this be the first parrot with nothing to say?

Mobile mayhem
Stop dialling and drive. An Australian study reports that using a cell phone while at the wheel of a car — even if it's on a hands-free device — quadruples the risk of having a crash.

NUMBER CRUNCH

A poll released last week suggests that President George W. Bush's policy on human embryonic stem-cell research doesn't match his country's mood.

63% of Americans say there should be a national policy for research using embryonic stem cells.

57% of Americans support using federal funds for embryonic stem-cell research.

59% of Americans would allow research into 'therapeutic cloning', a technique that can produce new stem-cell lines.

Source: Research!America

Caveman DNA hints at map of migration

The oldest sample of human DNA ever isolated in the Americas is providing a glimpse of how people spread across the land masses.

The DNA was extracted from teeth, more than 10,000 years old, found in a cave on the northern tip of Prince of Wales Island, off southern Alaska. Researchers compared the pattern of mutations in the DNA against those in thousands of samples. They found matches with 47 Native Americans from tribes living in areas ranging from North America to Tierra del Fuego, showing how the caveman's descendants must have spread.

“I could hardly believe what I found,” says molecular anthropologist Brian Kemp from the University of California, Davis, who reported the results last month at a regional meeting of the American Association for the Advancement of Science in Ashland, Oregon.

The work, which has yet to undergo peer review, indicates how genetic techniques are giving researchers a new window on genetic migration, says Kemp. Cooperation between scientists and Native American tribes on the project is cited as an example of how science and traditional cultures can coexist — in stark contrast to the case of Kennewick man, bitterly

disputed for nine years before studies finally began last month (see *Nature* 436, 10; 2005).

The DNA was extracted from teeth on a mandible found in 1996 in On Your Knees Cave, named by the explorer who first crawled inside in 1993. Carbon dating in 1997 showed the remains to be from someone living 10,300 years ago. Attempts to extract DNA from the bone failed, so Kemp spent two years probing the teeth. Working in high-containment facilities to prevent contamination, he and his colleagues finally managed to isolate fragments of mitochondrial DNA, which is passed down the maternal line, and Y chromosome DNA, which is passed from father to son.

The researchers compared the mitochondrial DNA with nearly 3,500 Native American sequences available in public databases. They found 47 matches, mostly from modern individuals but some from samples up to 1,500 years old. More than half of the matches were with members of the the Cayapa coastal tribe in Ecuador. Others were with members of the Chumash tribe of California, the Klunk Mound people in Illinois, the Tarahumara of Chihuahua in Mexico and the Mapuche and Yaghan tribes of Chile.

The caveman belonged to 'lineage D', one of the five founding lineages believed to have settled in the Americas more than 10,000 years ago. Lineage D is thought to have originated in Asia, and researchers also found a close match with a member of the Han ethnic group from Qingdao in eastern China.

“This is an exciting frontier,” says co-author James Dixon, an archaeologist at the University of Colorado, Boulder, who first dated the remains. But he adds that it will be important to repeat the work with other samples.

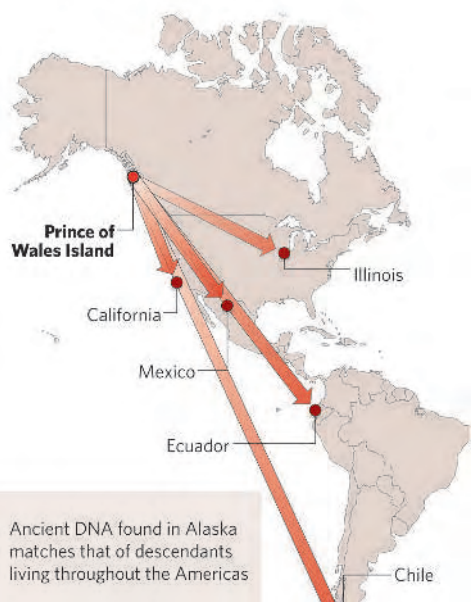
The local Tlingit and Haida tribes welcomed the project, which supports their concept of 'haa shagoon', learning from their ancestors.

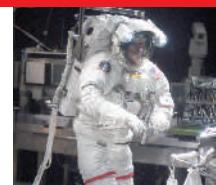
“We went right to the tribes within 24 hours of finding the bones, asking them to be a partner,” says Terence Fifield, the US Forest Service archaeologist for the region.

“We viewed the remains as offering us knowledge,” says Rosita Worl, a Tlingit tribal member who is a Harvard-trained cultural anthropologist. “We wanted the knowledge for current and future generations.”

Rex Dalton

CROSSING CONTINENTS



**SPACE SHUTTLE**

Read all about NASA's return to human spaceflight at

www.nature.com/news/specials/returntoflight

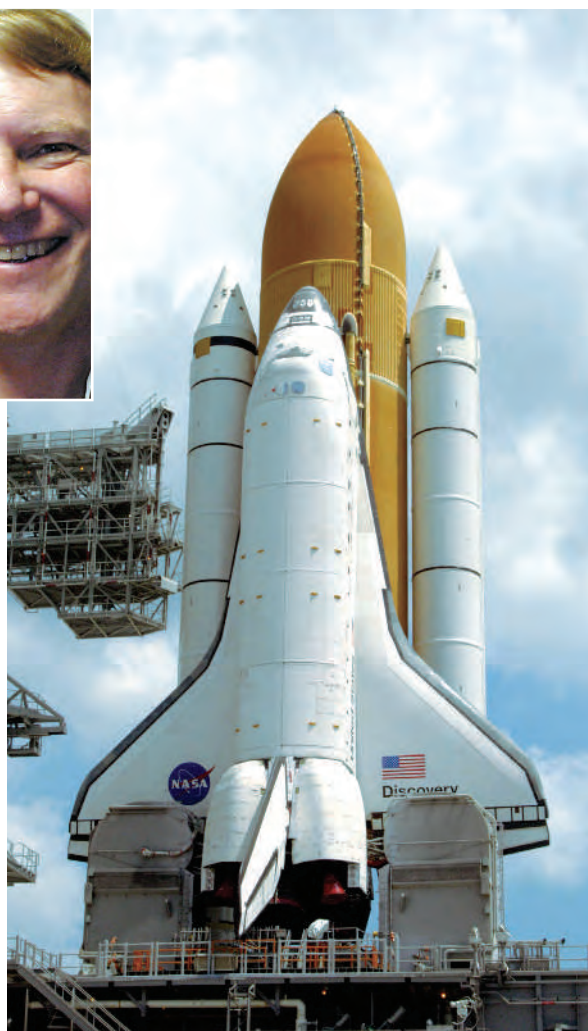
NASA

NASA/KSC; K. SHEEHAN

Time to move on from shuttle, says astronaut

As *Nature* went to press, the space shuttle *Discovery* was poised for a 13 July launch in the first shuttle mission since *Columbia* burned up on re-entry in February 2003. NASA managers have done their best to fix the problems that led to the disaster, but the 21-year-old vehicle still faces significant risks.

Three-time shuttle astronaut **George 'Pinky' Nelson** had the unique experience of flying the missions immediately before and after the 1986 *Challenger* accident. An astrophysicist, he left NASA in 1989 and now directs the Science, Mathematics and Technology Education programme at Western Washington University in Bellingham. *Nature* asked him how it felt to be one of the first astronauts flying the shuttle after a disaster, and what he thinks of NASA's plans.



Blast from the past: George Nelson knows the shuttle's dangers.

What was different about the first flight after the accident? Had your perception of the risk changed?

No. Actually, the flight before *Challenger* was really ragged. We had five launch attempts and all kinds of technical problems. You could see things weren't going well, but when you're in the cockpit, you just want to fly. The flight after *Challenger*, we knew that bad things can happen, but I felt that they had looked at everything twice and it was going to be a pretty safe mission. I was confident that if something got us, it wouldn't be the O-rings.

And it probably won't be debris striking the wing next time.

Yes, that was really a fluke. But there's no excuse for flying when they knew the O-rings were burning or before *Columbia* when they knew they were losing material off the external tank. I don't fault the engineers, I fault management. Falling debris is a problem that got them once, and if they haven't fixed it, they shouldn't be flying.

Do you think things have really changed with the shuttle programme?

No. The shuttle's a dangerous machine. It should have been put to bed ten years ago. I mean, it's a marvellous machine, but it never worked out to be the operational

spacecraft that they hoped it would be, and they should have moved on.

Are you happy with plans to build a Crew Exploration Vehicle for the Moon and Mars?

Yeah, I don't think much of the space station, and it will be nice to have another vehicle. The station is a marvellous engineering achievement, but it was sold as something else. There's no science being done. If anything, they should have built a platform around the Moon or somewhere interesting.

What about the current astronaut corps? There aren't many flight opportunities, but there are around 100 active astronauts.

That's something NASA should think hard about. How many astronauts do they really

need for what they're trying to do? They still have the skill mix for their initial fantasies about the shuttle and space station. I feel sorry for those guys — the world's most talented people just going to meetings, sweating, living in the world's worst climate [in Houston, Texas]. I don't know what I'd do if I were in their shoes. There are still interesting things for an astronaut to do, though. Building the space station's got to be a great mission. And servicing the Hubble Space Telescope — I think [NASA administrator Michael] Griffin will make a good call on that.

What is the right call?

If you're going to fly the shuttle at all, you ought to service the space telescope. Here's something you can do to contribute to science — it's got to be the number one priority. I had no respect for [former administrator Sean] O'Keefe's decision-making on that. He got burned by *Columbia*, and was trying to get rid of every mission he could. I don't think he realized NASA was an agency that actually did things.

Can NASA be revitalized?

I don't know. Right now, NASA is getting by on inertia. There are NASA centres all over the country, money's being spent and

the aerospace industry needs to employ people — that's what's driving it all. They have no mission, like saving the world from Communism or whatever else has driven the space programme in the past. I would like to see the Moon-Mars exploration programme happen. But we're spending a lot of money blowing up stucco in Iraq. It's hard to send aluminium into space when you're sending bricks and mortar 50 feet up.

You're focusing on education these days. Has space exploration lost its unique ability to inspire schoolchildren?

I think so, although science is still a motivator. Space has its place, but it isn't necessarily what everyone wants to do any more. ■

Interview by Tony Reichhardt

Japan's 'red bird' telescope flies into Earth orbit

It took two tries, but Japan finally launched a long-awaited X-ray telescope into orbit on 10 July. The Astro-EII mission is a replacement for the original Astro-E satellite, which was lost after a rocket failure during its February 2000 launch.

Astro-EII soared into space from the Uchinoura Space Centre on Kyushu Island aboard an M5 rocket. Once the spacecraft had reached orbit, the Japan Aerospace Exploration Agency announced that it had been given the nickname Suzaku, which means 'red bird of the south'.

Mission controllers will now test its five X-ray telescopes, which are designed to detect radiation coming from the matter swirling around black holes and other energetic cosmic phenomena. Suzaku's findings will complement those of two other major X-ray satellites, NASA's Chandra X-ray Observatory and the European Space Agency's XMM-Newton mission.

Rising tide of polio leads to pledges of extra funding

Polio is spreading in Yemen and Indonesia — countries once free of the disease — even as global leaders redouble their efforts to fight these and other polio outbreaks.

Yemen, which now has 300 polio cases according to the World Health Organization (WHO), was due to begin a large vaccination campaign this week. In Indonesia, at least 122 people are infected, and the government plans a push to vaccinate more than 24 million children in August, UN officials report.

Last week the UK government pledged

IMAGE
UNAVAILABLE
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Fight-back: Yemen is stepping up polio vaccination

£60 million (US\$108 million) to the WHO's polio eradication campaign, an amount that would more than fill the initiative's 2005 budget deficit. Leaders of the G8 nations, in their statement on Africa issued after their summit last week, agreed to work towards finding the funds necessary to meet the campaign's budget from 2006 to 2008.

Lab monitoring ecology of nuclear site faces closure

Budget cuts are threatening to shut down a respected US ecological research facility. The Savannah River Ecology Laboratory in South Carolina is laying off nearly a third of its scientific staff and may be closed permanently if more money cannot be found.

For 50 years, the research laboratory near Aiken has monitored the environment around the US Department of Energy's Savannah River nuclear plant, where five now-deactivated reactors generated plutonium and tritium for bombs. But in the spring, the Bush administration moved to cancel the department's US\$8 million contract with the University of Georgia to operate the facility next year.

Regional Congressmen have cobbled together about \$4 million for the lab in

2006, but 50 of the approximately 150 scientific positions have been axed. Many staff are looking for jobs elsewhere. "It is very disturbing," says Whitfield Gibbons, a snake expert at the lab.

K. FAZAA/AFP/GETTY

Signatories widen scope of nuclear materials treaty

The world's only legally binding agreement to monitor the whereabouts of nuclear materials may soon have a wider scope. On 8 July, a majority of the member states of the Convention on the Physical Protection of Nuclear Material agreed to amend the convention to include nuclear facilities and material for peaceful domestic use, both in storage and while it is being transported.

The convention, created in 1980, previously applied only to nuclear material in international transit. To come into effect, the new rules will have to be ratified by two-thirds of the convention's 112 member states. The process could take several years, according to a spokesman for the International Atomic Energy Agency based in Vienna, Austria.

Cancer-vaccine researcher censured over misconduct

After an investigation lasting almost three years, a German researcher was last week held to be responsible for serious scientific misconduct.

In 2000, Rolf-Herrmann Ringert, head of urology at the University of Göttingen, co-authored a *Nature Medicine* paper that claimed a significant advance in cancer vaccination: that patients' kidney tumours regressed after they were vaccinated with a cocktail of tumour cells fused with immune cells (A. Kugler *et al. Nature Med.* 6, 332–336; 2000). An internal university investigation found the paper's first author, Alexander Kugler, guilty of negligence, while clearing Ringert and all the other co-authors (see *Nature* 420, 258; 2002).

But the DFG, Germany's main research funding agency, carried out its own investigation and came to a harsher conclusion. It argues that as corresponding author and head of the urology department, Ringert was responsible for the false methods, data and claims in the paper. He is now barred from applying for DFG grant money, acting as a referee for the agency, or participating in DFG elections for the next eight years.

In an unrelated case, the DFG announced on 5 July that it had cleared molecular cardiologist Stefanie Dimmeler — winner of the agency's €1.5-million (US\$1.8-million) Leibniz Prize — after anonymous allegations of scientific misconduct (see *Nature* 434, 130; 2005).

D. KOROTAYEV/AP PHOTO

IMAGE
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Camera millionaire snaps up a week's break on space station

Just don't call him a space tourist: a rumoured US\$20 million has bought Gregory Olsen a ticket to the International Space Station, but unlike previous visitors Dennis Tito and Mark Shuttleworth, he prefers to be called a 'private researcher'. Olsen, an American who founded companies that make electronic imaging equipment, says he plans to "do a lot of science up there". Having signed a contract on 6 July with the Russian space agency, he

could fly to the station in October for eight days.

One of his experiments will reportedly grow semiconducting crystals of the type used in his company's products, which include infrared cameras used for night-vision equipment.

Olsen says he will also use a miniaturized camera to observe crops from space, look at the effect of pollution on the atmosphere and conduct near-infrared astronomical studies.



HARD GRAFT

Surgeon Fiona Wood has pioneered a controversial treatment to reduce scarring in burns victims.

Carina Dennis finds out how she counters her critics.

Fiona Wood is in a rush. Colleagues say she wants everything yesterday. A master of multitasking, she travels exhaustively and handles a hefty clinical and research load. It is this boundless energy and sense of urgency that drove the surgeon to develop a pioneering treatment to help burns victims.

Originally from Yorkshire, UK, Wood now heads a burns unit at the Royal Perth Hospital in Western Australia, where she has developed a technique called 'spray-on skin'. It literally involves spraying a patient's wounds with their own skin cells, and works much faster than conventional methods. Wood says this speed helps minimize scarring and accelerates recovery.

Her method was put to the test under horrific circumstances in October 2002, when victims of the Bali terrorist bomb were flown to her unit. Her team worked around the clock, and managed to save all but three of the 28 patients who arrived. The incident propelled Wood into

the media spotlight and she was named Australian of the Year 2005.

But fellow surgeons and scientists argue that her spray-on skin hasn't been proven to work. Unless it is subjected to rigorous clinical trials, it will never become accepted clinical practice, they say. Unfazed, Wood says her method is a key step towards scarless healing and will be useful in other routine surgical procedures, such as scar repair and cosmetic surgery.

Burns can be truly gruesome injuries. Without skin, the body weeps copious fluids and is dangerously vulnerable to infection. If a patient survives, they face living with disfiguring scars that tighten and restrict movement. Although Wood is very matter-of-fact when she talks about burns, she is still overwhelmed by the suffering of her patients,

IMAGE
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Tough challenge: several victims of the Bali bomb (above) were given spray-on skin grafts by Fiona Wood (top).

especially children. "The day you stop being affected, is the day you should hang up your boots in my opinion," she says.

How a burn is treated depends on the extent of the damage to the two layers that make up our skin. The upper epidermis contains keratinocytes, the cells that make keratin, which

ROYAL PERTH HOSP.

forms a tough protective sheath. The lower dermis is composed largely of cells called fibroblasts, which make collagen, giving skin its durability and elasticity. Shallow burns that singe the epidermis are left to heal on their own. But when the burn is deeper and more extensive, skin grafts are used. Thin strips of the patient's healthy skin are shaved off a donor site, taking mostly epidermis and a little dermis, and stretched over the exposed wounds. For severely burned patients, donor sites often need to be reharvested, but they usually take up to two weeks to grow back. An alternative is to use sheets of the patient's cultured skin cells, but these take three weeks to grow.

Treating severe burns is a race against time. "Seventy-five per cent of burn scarring remains permanent if it's not healed within 21 days," says Wood. It also becomes a numbers game: the more burns there are, the less healthy skin there is available to harvest and cover the wounds. To buy time, surgeons can use coverings made from cadaver skin or manufactured sheets of foreskin cells. "But as these do not contain the patient's cells, they do not always result in permanent wound closure," says Wood.

Driven to act

A key turning point for Wood came in 1990, when she was part of a team caring for a patient with severe burns. The group was experimenting with the then-new technique of growing sheets of skin cells derived from the patient. The cells had just begun to 'take' when the patient — who had survived for seven months — died from an infection that had invaded while her skin was missing. "I was overwhelmed by the thought that we had to be able to do better," says Wood. "If we'd been quicker, the outcome might have been different."

A few years later, she did do better. Wood recruited Marie Stoner, a cell biologist, and together they reduced the time for culturing a patient's skin cells from weeks to just five days. There was no magic, says Wood; rather, they simply whittled down the time to grow skin cells and sprayed them onto the burn using a syringe with a spray nozzle attached.

Wood's method — which she dubbed CellSpray — involves taking a skin sample about the size of a postage stamp, scraping off the keratinocytes and multiplying them in tissue culture for several days. The cells are then harvested and sprayed onto the wound¹.

But CellSpray is not the whole solution. Wood often uses it in conjunction with traditional grafts. Keratinocytes need a healthy underlying dermis as an anchor point and source of nutrients. So if the burn has damaged or destroyed the dermis, Wood uses a skin graft before spraying it with the cultured cells. If the dermis is lost completely, it first has



Fiona Wood hopes to find a way to heal wounds without leaving scars.

to be rebuilt using either a much thicker skin graft or an artificial protein scaffold into which fibroblasts and other cells migrate. After a few weeks, the epidermis can then be added over the top by grafting as well as spraying.

CellSpray was first used on patients in 1995 and has been routinely deployed in Wood's burns unit ever since. She is adamant that it leads to faster healing and reduces scarring. It also requires fewer donor sites — particularly useful for severely burnt patients.

But Wood's peers are sceptical. Peter Haertsch, head of burns and reconstructive surgery at Concord Hospital in Sydney, Australia, says it's a brilliant idea but argues that there is not enough evidence that it works.

Haertsch is strongly critical, even though he is experimenting with the technique in his

"The day you stop being affected, is the day you should hang up your boots." — Fiona Wood

own unit. But he is not alone — the burns specialists interviewed for this article agreed that randomized clinical trials are needed. There is also some doubt over whether the spray-on cells fall off over time. For this reason, Haertsch says it's unclear how much the spray actually contributes to wound healing compared with the underlying skin graft and cells migrating in from surrounding tissues.

But Wood believes her results speak for themselves. "The evidence is borne out by experience," she says. She and her staff are convinced that patients suffer significantly less scarring thanks to the technique. As a result, she feels it would be unethical to do clinical trials in which some patients get spray-on skin whereas others get conventional grafts. Furthermore, it is difficult to compare burns treatments because of the variability of the wounds.

Wood has published some results using spray-on skin in animal models^{2,3}, but she acknowledges that she won't convince her peers without clinical trials and has helped to design a multicentre randomized trial to be conducted

in Australia and internationally. This will involve patients who have burns to more than 20% of their body. Each patient will receive traditional skin grafts on one side of their body and spray-on cells plus a skin graft on the other side. Wood and her team are conducting a complementary study in which they are comparing the outcomes for their patients with those at other units who have received conventional treatment.

While experts ponder its value, Wood has ambitious plans for CellSpray. She thinks it could also be useful for minimizing scarring in routine surgical procedures

such as repairing birthmarks or removing moles. A kit for the method, called ReCell, is being distributed by Clinical Cell Culture, a company that Wood co-founded in 1999. It has been submitted for approval by regulatory agencies including the US Food and Drug Administration and its Australian equivalent, the Therapeutic Goods Administration, and has recently been approved for clinical use in the European Union and Japan.

Reluctant entrepreneur

Wood says that commercializing her work has been an "educational experience" but adds that she doesn't feel comfortable in the role. Rather, she sees it as a means to raise funds for the non-profit McComb Foundation, which supports burns research using her company's profits.

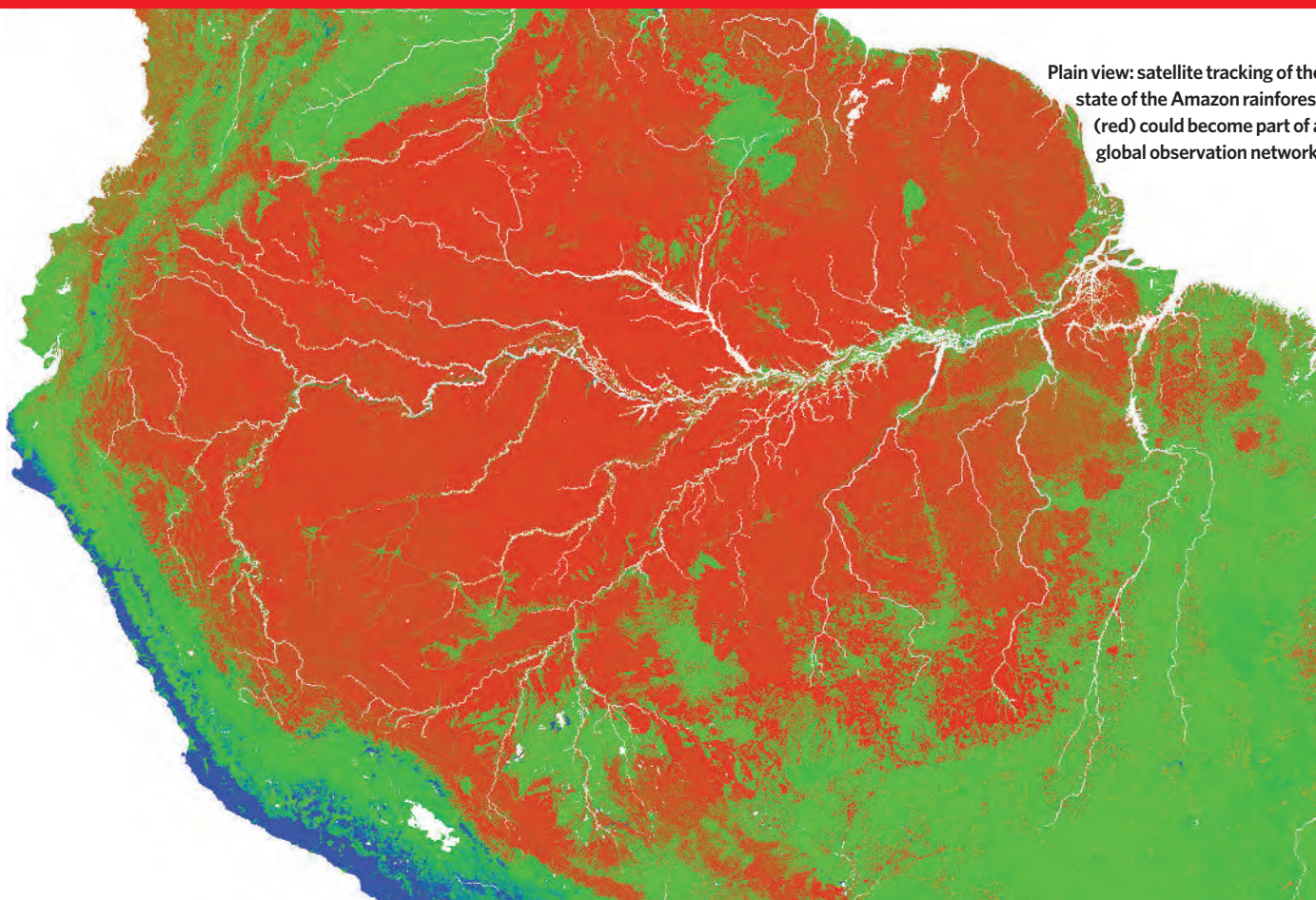
Ultimately, Wood wants to achieve healing without scars. She is now trying to combine the reconstruction of the dermis and epidermis into a single step. To do this, she sprays keratinocytes onto Integra, an artificial scaffold derived from shark cartilage and cow collagen. This is then applied to the wound, where the dermal cells move into the scaffold to regenerate the dermis, and the keratinocytes migrate to the top to rebuild the epidermis. Wood's team claims success with a mouse model and hopes to test the technique in patients soon.

The team is also trying to improve artificial scaffolds and is using three-dimensional scans to build a picture of skin topography, burns and the healing process at different sites of the body. The ultimate goal is to use computer technology to scan patients and predict the best treatment for different burns. Wood adds that this will help to quantify the outcome of her treatments, and hopefully appease her critics.

Although she is an enthusiast of basic research, Wood's clinical pragmatism never subsides. "It might be nice science, but I need to be able to use it," says Wood. And the sooner, the better.

Carina Dennis is Nature's Australasian correspondent.

1. Wood, F. M. *Wounds* **15**, 16–22 (2003).
2. Navarro, F. A. et al. *J. Burn Care Rehabil.* **21**, 513–518 (2000).
3. Navarro, F. A. et al. *J. Burn Care Rehabil.* **22**, 41–46 (2001).



Plain view: satellite tracking of the state of the Amazon rainforest (red) could become part of a global observation network.

SOMETHING TO WATCH OVER US

An ambitious international project to unite the planet's Earth-observing systems is under way. But getting everyone on board is no easy task, says **Naomi Lubick**.

Earth has no shortage of spies — mechanical ones, that is. A range of instruments, including satellites, tide gauges and weather stations, constantly track natural phenomena. But like human spies, these machines rarely speak to each other — hampering the efforts of scientists, politicians and planners to best manage the planet's natural systems.

It took a horrifying natural disaster to put Earth observation in the public consciousness. Soon after the Indian Ocean tsunami killed nearly a quarter of a million people in December 2004, news reports revealed that some of the hydrophones, seismometers and other devices that could have provided a warning were in place — they just weren't linked together in real time to provide an effective alert.

Now plans are afoot for a system to warn of future tsunamis in the Indian Ocean and, with little public fanfare, a much more ambitious scheme is taking shape. The Global Earth Observation System of Systems, or GEOSS, promises global, instantaneous hook-up of

satellites, ground-based stations, and oceanographic and atmospheric instruments. Within a decade, if all goes according to plan, scientists from participating countries will share data on a variety of global phenomena — from sea-surface temperatures, through rainfall totals, to land-use patterns.

GEOSS would link the more than 50 Earth-observing satellites with thousands of oceanographic buoys and terrestrial observing stations. Scientists could combine millions of data sets into one coherent picture, which planners could use to improve people's lives. Using data from anywhere in the world, researchers would be able to predict the long-term agricultural outlook for various regions, track wetlands that are threatened by development, or send alerts when standing water is plentiful enough for the breeding of bumper populations of malaria-bearing mosquitoes.

But GEOSS faces many procedural stumbling blocks. It's not clear what kind of information should be shared or by whom. Money

remains a major issue in a field where funding for long-term monitoring projects is often slashed. The United States has coughed up most of GEOSS' start-up costs so far — up to US\$750,000 in two years. Now that the programme has moved its headquarters from the United States to Geneva, other governments are starting to contribute money and staff.

Good connections

Scientists and policy-makers had talked about something like GEOSS for decades, but plans only began to crystallize at the 2002 World Summit on Sustainable Development in Johannesburg and at the 2003 G8 summit in Evian, France. Now supporters are busy identifying data gaps, and hope to put new instruments in place to fill them within the next four years. By 2013, advocates hope to have a complete data-sharing system whose output could help people make better-informed decisions about their lives. One day, GEOSS products could help forest managers track erosion, climatologists follow dust storms, or even anglers

find the best spot to cast their line.

On some levels, scientists already share their observations of Earth. Researchers have long swapped information from remote sensing satellites such as the US Landsat series and the European Space Agency's ERS satellites. GEOSS is a natural extension of this. Most scientists agree that the billions of dollars that already flow into Earth observing are not always spent most effectively. Data sets overlap dramatically for some observations, such as meteorological satellite recordings, but are sorely lacking in others, such as hydrophone measurements that could warn of tsunamis. And earlier international efforts to coordinate observations, such as the Global Ocean Observing System and the Global Climate Observing System, suffer from funding shortages.

Global vision

Yet since the Johannesburg summit, high-level political backing for GEOSS has coalesced relatively quickly. In 2003, ministers from 33 countries and representatives of the European Union (EU) attended the first global Earth Observation Summit in Washington DC. Interest swelled and by February this year, 58 governments had signed a cooperative agreement in Brussels. Such international attention could keep the pressure on individual governments to stay with GEOSS, says Don Hinsman, director of the space programme at the World Meteorological Organization (WMO) in Geneva. "That type of visibility will only ensure continued support," he says.

But some countries are hesitant to climb on board. For instance, Tanzania's government has yet to consider joining. Mohammed Mhita, director-general of the Tanzania Meteorological Agency, and other supporters are working out how to encourage the financial investment for computing power and high-speed telecommunications needed to take full advantage of GEOSS. They are also discussing which organization should take the lead on

national efforts. And in Saudi Arabia, some scientists have spoken of difficulties in getting data from their own country's sources. Secretive governments may not want to share their data but "it becomes harder to hold out in an interconnected world" says Joanne Gabrynowicz, a US delegate to several recent GEOSS meetings and the director of the National Remote Sensing and Space Law Center of the University of Mississippi.

Other nations may feel they could lose their self-sufficiency if they share too much, says John Zillman, president of the Australian Academy of Technological Sciences and Engineering, and former president of the WMO. Under GEOSS, he notes, a researcher could sit in the United States and produce forecasts for regions thousands of miles away. "How is the national meteorological survey of Nigeria going to feel?" he asks. "Are they going to be happy to see their data flowing freely, while you in the United States perform their forecasts? That's the dilemma."

Such issues are also a concern for emerging world powers. India, which has a strong telecommunications and scientific infrastructure, has been reluctant to share data from its network of seismometers. Because the information is considered vital to national security, the government prevents it from being sent out in real time — a serious potential problem for the fledgling Indian Ocean tsunami warning system. Some data are held indefinitely as they may pertain to nuclear testing. And economic considerations also come into play: satellite images and other kinds of high-resolution data are sometimes reserved for sale.

In many cases, GEOSS might allow countries to keep sensitive data to themselves, while sharing information that could benefit other countries. "We look at GEOSS to contribute, but we also look at it to benefit us in areas

where we don't have missions," says Mukund Rao, a geologist at the Indian Space Research Organisation and president of the Global Spatial Data Infrastructure Association. For instance, India has no ocean observations that track sea-surface height and temperature and so might be interested in acquiring them through GEOSS.

But even the most dedicated countries, such as the United States, cannot guarantee their long-term support. Several US Earth-observing projects — such as the collection of national measurements of streamflow by the US Geological Survey — are losing funding.

And the US government only recently made plans to launch the successor to the Landsat 7 remote sensing satellite. Now, there may be a potential gap of several years before the next-generation Landsat is launched.

GEOSS' birth pangs might be lessened, say observers, if the pro-

gramme models itself on the WMO. With 187 member countries and territories, most with their own meteorological division, the body has had great success in sharing databases. It took years for countries to make sure their data were in the correct format for sharing, and to figure out how to get the information to each other, says Zillman — but they did it in the end. GEOSS is already taking its first steps in this direction: at a meeting in May, participants agreed in principle to accept carefully worded language on how to retain control over data while pledging to share them.

For now, GEOSS is housed at the WMO's offices in Geneva, under an independent coordinating secretariat. Some observers think GEOSS should be moved under the auspices of the United Nations (UN), whereas others wonder if it should remain an independent organization, much like the Intergovernmental Panel on Climate Change.

"There are arguments for both," says Vice Admiral Conrad Lautenbacher, head of the US National Oceanic and Atmospheric Administration and a driving force behind GEOSS. In its current independent state, GEOSS "doesn't have the huge bureaucracy, procedures and baggage that you have as a formal UN organization" Lautenbacher says. Instead, the relatively informal coalition allows parties to talk to each other in a non-threatening forum. On the other hand, although the UN infrastructure may be unwieldy, the UN provides a venue where countries are used to dealing with each other, which might be important for building trust, says Zillman.

However it ends up being structured, GEOSS has the potential to reshape the face of Earth observing supporters say. If the early momentum can be sustained, the spies will start sharing crucial observations. Earth science will benefit from the silence being broken.

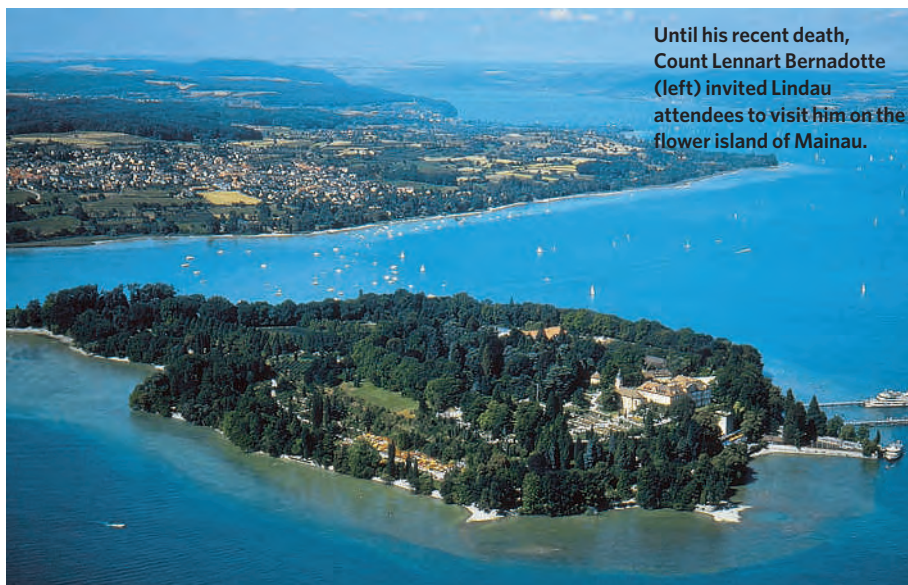
Naomi Lubick is a reporter at *Geotimes* magazine.

However it ends up being structured, GEOSS has the potential to reshape the face of Earth observing.



Under GEOSS, data would be shared from streamflow gauges such as this one on Snake River in Wyoming.

MAINAU



Until his recent death, Count Lennart Bernadotte (left) invited Lindau attendees to visit him on the flower island of Mainau.

MAINAU

CLOSE ENCOUNTERS

After a low-key existence for more than 50 years, Germany's Lindau meetings have opened their doors to the world. **Alison Abbott** joined 44 Nobel laureates as they mingled with young scientists.

For the past 55 years, a remarkable gathering of minds has been taking place in a small town in Germany. Behind closed doors, Nobel laureates have come from all corners of the globe to meet local students. This year, for the first time, the rest of the world has been invited and some 700 international students have joined the party.

"I can't believe my luck," says Evan Thomas, a chemist working on his PhD in Louisiana State University in Baton Rouge, on the first evening. "I don't know what I'm going to say to them, but I'll definitely say something having come all this way."

Qi Zhang, a PhD student from the Fourth Military Medical University in Xi'an, China, is bubbling over with excitement following a chat with 1978 medicine laureate, Werner Arber. "He discovered restriction enzymes ... what we use in the lab all the time... we couldn't work without them... I was talking with him... I can't believe it."

The story of these unusual gatherings begins in postwar Germany. It involves a count, a casino and the small medieval island town of Lindau on Lake Constance. And it has a happy ending: after years of financial struggles, the 2005 Lindau meeting has made the switch from parochial to international, has put its funding on a firmer footing and is using English — for the first time — as its official language.

"The meetings very much needed to become international," says Arber, a regular Lindau attendee. "The German students attending needed that boost — they were becoming complacent." And times have changed, he notes,

since the meeting was conceived in 1949.

Back then, Lindau was under French occupation. It was an unlikely time and place to plan an international gathering of Nobel laureates: Germany was destroyed as a scientific, as well as a political, force.

Despite this, three local personalities, who saw science as a promoter of 'peace and brotherhood', managed to bring laureates to Lindau. The original idea belonged to Gustav Parade, a doctor at the Lindau district hospital, who wanted to do something about the scientific isolation of Germany in his field. He teamed up with a local gynaecologist, Franz Karl Hein, to propose inviting Nobel laureates in physiology or medicine for discussions with doctors and scientists from around Germany.

In for the count

Emboldened by the backing of the mayor of Lindau, in early 1950 the two men boated across Lake Constance to the tiny 'flower island' of Mainau. There they sought the support of its owner, Count Lennart Bernadotte, great grandson of King Oscar II, who presented the first Nobel awards in 1901.

The count did not hesitate, and his patronage became the hallmark of the annual meetings until he died last December at the age of 95. Tireless in his dedication to the cause he would stand in line with autograph hunters at the Stockholm Nobel award ceremony for the opportunity to

persuade new laureates to take part in the Lindau meetings.

In the early days it was hard to find cash within Germany's ruined economy for such an indulgence. But political confusion came to the rescue. Being the only corner of Bavaria not under US occupation, Lindau enjoyed a special administrative status that allowed it to sidestep gambling laws. Soon, the town established the Bayerische Spielbank Casino, which became a major source of funding for the meetings.

Attracting Nobel laureates turned out to be the easy part. The first European Meeting of Nobel Laureates in Medicine, in June 1951, was attended by six German laureates and one from the United States as well as some 400 physicians. It was deemed by all to be a big success, although the Swedish Nobel Foundation, which saw Stockholm as the laureates' only legitimate home base, was unimpressed. Tensions have eased in recent years, but the foundation has never formally endorsed the series.

During the first 50 years, a rhythm was established, with meetings rotating between medicine, chemistry and physics. Students, mostly German undergraduates selected according to academic performance, soon replaced the physicians of the first meeting. They

"Nobel laureates are like the rest of the world — some are smart, some are average and some are dumb." — Ivar Giaever

enjoyed not only morning lectures, but also the informal company of Nobel laureates during afternoon discussions, a dinner and dance on the first evening, and the traditional final-day boat trip to Mainau as guests of the count.

The laureates quickly came to love the formula, and many come as often as their schedules allow. "I must hold an attendance record," boasts Ivar Giaever, who won the physics prize in 1973 and has been to every physics meeting since. "They make me feel like a king!"

But the series has been on the verge of financial collapse several times. Federal and regional governments came to the rescue in 1970, but it was not until the 50th meeting in 2000 that a formal foundation was established to put it on more secure ground.

And despite their old-world charm, the Lindau meetings had become an anachronism. Germany was no longer scientifically isolated, and the privileged access to laureates was harder to justify. The failure to recognize English as the international language of science reinforced the air of provincialism.

In the past few years, some laureates became worried that unless they opened up to the world, new laureates would not make the time to come. At the same time Count Lennart was ageing — and he was keen to ensure continuation after his death.

Glittering prize

From 2001, the new foundation's 14-strong committee began to sort out the meetings' finances, and to make them more relevant to science as practised today. They invited scientific academies and other agencies around the world to open competitions for young scientists to attend, then whittled down a list of nearly 10,000 applicants. The final 2005 list of 720 invitees represented a new profile of participant: academically excellent, familiar with societal impacts of their research and fluent in English. They are generally under 30, but the majority are now PhD students or postdocs, pushing up the general level of education.

The weekly schedule of the meetings remains the same, although the lectures are also broadcast live on the Internet. And economics now takes its turn — the first such gathering last year attracted 11 economics laureates. And as of this year, medicine, chemistry and physics will all meet together every five years.

Some things haven't changed — most notably curiosity about Nobel prize-winners as human beings. Even the laureates confess to curiosity — and sometimes reverence — for each other. "I met Dirac once," recalls Giaever with relish, referring to Paul Dirac, the legendary, but famously withdrawn, 1933 physics laureate who theorized the existence of antimatter. "He was very quiet, very quiet."

Some students entertain hopes that they may be recruited into a Nobel lab, as has happened on several occasions. Others long for insight into a secret formula that could help their own quests for

success. "I want to learn from them how to think: what is the right thought process and where do you begin," says Steve Bull, a doctoral student in chemistry at Northwestern University in Evanston, Illinois.

Another student wanted to know if the laureates thought that they had simply been in the right place at the right time. "There is a bit of

that," admits Günter Blobel (medicine, 1999). "But it isn't the whole story." Giaever sees more diversity among his fellow laureates: "Nobel laureates are like the rest of the world — some are smart, some are average and some are dumb."

Blobel learned something, in turn, about the young scientists. "It is curious to see the questions that students from different cultures ask," he remarked after a discussion on evolutionary biology led by Christian de Duve (medicine, 1974). He was taken aback to find some students expressing so much interest in the 'creative guiding hand' of intelligent design.

Political science

Past Lindau meetings have had politics as well as science on the agenda. Back in 1955, some 18 laureates who had been involved in nuclear research, including Werner Heisenberg (physics, 1932), were invited to the chemists' meeting. There they signed the 'Mainau declaration', which called on governments to abandon the development of nuclear weapons, and played an important role in the 1950s ban-the-bomb campaigns.

Since then, many laureates, most prominently Albert Einstein (physics, 1921), have used their fame to push political agendas, mostly promoting peace or equality. Just last month, 18 laureates signed a petition calling for stronger political leadership on neglected diseases.

Several at the 2005 Lindau meeting say they have launched petitions of their own. Others say that they prefer to stay out of politics altogether. "I know nothing more than any man on the street and have never signed a petition that identified me as a Nobel prizewinner," says Robert Richardson (physics, 1996).

But politics is never entirely forgotten in Lindau. In their lectures, many laureates recall their personal debts to Jewish scientists driven from Hitler's Germany to seed frontline research in the United States and elsewhere.

And the philosophy of the new-style meetings is to support excellence in science wherever in the world political or social oppression may seek to stifle individuals.

Aside from such serious issues, Lindau exerts a simpler magic as well. "It's nice here," answered Masatoshi Koshiba (physics, 2002) when asked why he had come. "I just heard it was really good," said William Lipscomb (chemistry, 1976), whose wife added pointedly: "And I wanted to come."

Klaus von Klitzing (physics, 1985), who could be spotted rock-and-roll dancing with students on the first night, claimed it had always been his ambition to get in. He tried to get an invitation as a student, but was turned down. "So I knew I just had to win a Nobel prize if I wanted to attend," he smiles.

Alison Abbot is *Nature's* senior European correspondent.



Memories of a meeting: Ivar Giaever (top right) jokes with student Steve Bull; Werner Arber meets Qi Zhang.



Serious and social: chemistry laureate Kurt Wüthrich gives a lecture (top), and last year's physics medallist, Frank Wilczek (bottom right), heads for the dance floor.

BUSINESS

Industry lured by the gains of going green

Ecological conservation isn't exactly the number one priority in most corporate board rooms. But an international effort is under way to get large companies to start thinking seriously about ecosystems.

Next week, the Millennium Ecosystem Assessment — a global audit sponsored by, among other agencies, the United Nations and the World Bank — will release a 30-page report on why ecology matters in business planning.

The main millennium assessment report, released in March, stressed that more than half of the 'ecosystem services' that support human activities are being tapped in an unsustainable way. The new supplement argues that corporations should be preparing now for the depletion of those natural services.

It says that six major environmental trends — water scarcity, climate change, habitat change, biodiversity loss and invasive species, ocean exploitation and nutrient overloading — will each profoundly affect business. Resultant commercial opportunities exist in industries as diverse as energy, agriculture and tourism, the report contends.

"This is about providing a set of tools so that people can make better decisions," says Jonathan Lash, president of the World Resources Institute (WRI), the Washington-based environmental group that prepared the assessment reports.

Some critics argue that the new report is a missed opportunity, as it lacks specific examples that might entice industrial interest in ecosystem services. But the Geneva-based World Economic Forum, which represents 1,000 of the world's largest companies, says its members will find it useful for planning.

"For every risk, there's an opportunity for a producer to reduce that risk," explains Lash. For example, future regulation is likely to control the overuse of nitrogen fertilizers, run-off

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

Uproar in India forced Coca-Cola to revise its water-use strategy.

from which is triggering dead zones in the sea. This will create demand for better fertilization techniques, as well as for tools to monitor run-off.

But of all the resources associated with ecosystem services, it is water supply that promises to present the most volatile issues. Coca-Cola, for example, has already been banned from using groundwater for its bottling operations in India. To address the issue, and avoid negative publicity, Coca-Cola is preparing a plan for sustainable water use for all of its global plants. "As a hydration company, we have to do something," says Harry Ott, the company's director of

water resources, based in Atlanta, Georgia.

The need to address sustainability issues has "quietly crept onto the centre stage" over the past few years, says Jim Harmon, chairman of the WRI. But the authors of the report admit that there aren't many mechanisms in place to force action on ecological issues. Instead, they are counting on the emergence of market-based mechanisms, such as 'cap-and-trade' schemes to restrict pollution or encourage conservation.

The WRI plans to follow up the report by working with business to incorporate ecosystem services into such mechanisms. One established example in the United States has created an active market in wetland conservation: developers must procure and conserve areas of wetland to compensate for other areas they have built over.

Similar markets for water purification and biodiversity are steadily growing in scope, says Ricardo Bayon, managing editor of the Ecosystem Marketplace, a website based in Washington DC. Mexico and Malaysia, for example, have begun pilot programmes that will pay farmers to manage their land to protect watersheds. "We're at the tipping point," says Bayon. "We are going to see a lot more of these mechanisms used."

Virginia Gewin

J. FUSCO/GETTY IMAGES

IN BRIEF

PATENT DUMP The European Parliament has overwhelmingly thrown out a legislative proposal that would have streamlined the continent's existing patchwork of software patenting rules.

The parliament's 648-14 vote reflected widespread criticism that the proposal would have helped large companies to claim control of software concepts — hurting smaller firms and Europe's powerful open-source software movement (see *Nature* 435, 411; 2005).

The vote means that software patents in Europe will continue to be administered by national patent offices.

EXIT IONIX A one-time star prospect of the British biotechnology industry has been sold off to a rival firm. Cambridge-based Ionix Pharmaceuticals, whose main drug candidate is an opiate analgesic called IX-1003, was bought by Vernalis for shares worth about £12 million (US\$21 million).

Vernalis said it would close Ionix's lab at Cambridge Science Park, with the loss of 19 jobs, and take the company's products and technologies forward itself. Ionix had raised a total of about £24 million in venture capital since its foundation in 2001, but plans for a public offering of its shares never came to fruition.

VIOXX THUMBS-UP An advisory panel has told the Canadian health ministry to return the painkiller Vioxx to the market, following its withdrawal last September.

The drug was taken off sale by Merck, its manufacturer, after it was found to increase the risk of heart attacks and stroke. But the Canadian panel, echoing the findings of a US advisory panel in February (see *Nature* 433, 790; 2005), ruled that Vioxx's benefits outweighed the risks to patients. Health Canada hasn't yet said what it will do in response to the panel's findings.

The panel also ruled that Pfizer's Celebrex painkiller should stay on sale but that its arthritis product Bextra should remain unavailable. And it highlighted risks associated with established painkillers such as ibuprofen, which it said should only be sold over-the-counter by pharmacists.

Mysterious disappearance of female investigators

SIR — The results of the first year's European Young Investigator (EURYI) awards are worrying. The awards provide 25 young scientists with up to €1.25 million to establish research teams in Europe. Only three of the 25 initial recipients were women, far below the percentage working in science at the targeted career stage. This was not because women didn't apply in sufficient numbers: nearly a quarter of the applicants were female. Rather, male applicants were twice as likely to succeed.

EURYI applications were first submitted to the relevant national research councils, who could nominate a specific number of candidates. This selection cut the proportion of women from one-quarter to one-fifth. Each national research council oversaw a drop in the number of selected women. In Spain, where nearly a third of the applicants were women, not one was nominated. The all-male Spanish list emerged with the highest success rate in the later European rounds, nearly three times the average. From 133 national nominees, European evaluation committees created a shortlist of 67, causing the largest drop in the proportion of women: $9.9 \pm 0.5\%$ of men applying made the European shortlist, but only $4.7 \pm 1.4\%$ of women did.

The working paper *Evaluation of the EURYI Awards Scheme* by L. Langfeldt and K. E. Brofoss (NIFU STEP, Oslo, 2005), commissioned by the European Science Foundation (ESF), with access to a limited data set including assessments of candidates at the European evaluations and sample applications, recently concluded: "the main problem remains that the scheme ... attracts far fewer female applicants". The report also suggested that "female applicants had a somewhat higher tendency to be filtered out" at the domestic level but that there was "no evidence of bias" at a European level.

However, the statistics are clear: the consistent attrition of women at each stage, and the large size of the sample, mean that women's lack of success cannot have occurred by fluke. The random chance probability of halving the female fraction from one end of the competition to the other is only 0.05%.

The 'leaking pipe' phenomenon (in which a disproportionate number of women leave the sciences at each career stage) is often attributed to a complex array of external factors that cause women to drop out. In this case, we believe we are seeing a leaking pipe in the stages of a single competition. Does this mean more straightforward explanations for the career leakage may be possible?

Without a detailed knowledge of applications and judging criteria, it is impossible to nail down the underlying

reasons for the inequality in the awards. We would like to replicate the groundbreaking analysis of Christine Wennerås and Agnes Wold ("Nepotism and sexism in peer review" *Nature* **387**, 341–343; 1997) at the European level, but the ESF has so far been unwilling to release the necessary data to us.

We consider that this attrition demands further independent scrutiny to uncover the cause.

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Rising temperatures are likely to reduce crop yields

SIR — Your News story about the Royal Society meeting on climate change and food production ("Hikes in surface ozone could suffocate crops" *Nature* **435**, 7; 2005) noted that rising CO₂ levels will generally benefit crop growth, as this stimulates photosynthesis in most crop plants. However, the links between climate change and food production are even more complex than your story suggests.

Rising temperatures could extend the geographical distribution and growing season of some agricultural crops, such as pasture grasses, by allowing the threshold temperature for the start of growth to be reached sooner. This assumes that water and nutrients are supplied at a level that permits pasture crops to benefit from a longer growing season.

But in general, and contrary to common perceptions, most crop physiologists expect global warming to reduce crop yields. This is because higher temperatures shorten the life cycle of most cereals, hastening senescence and reducing the length of the growing season. Other effects such as an increase in tropospheric ozone level can exacerbate crop senescence, as noted in your News story. The staple cereal crops can only tolerate narrow temperature ranges, which, if exceeded during the flowering phase, can damage fertile seed production and thus reduce yield.

Global warming would also be expected to increase the frequency of exposure to extreme temperatures and thus damage crop fertility.

So far, efforts to predict climate change effects on food production and quality have been fragmented. For major crops, except wheat and soybean, we lack the agronomic-scale experiments needed to understand and robustly predict the direct effects of CO₂ and ozone, and their interactions with temperature and water. With an extra three billion people to feed during the coming 40 to 50 years, closer cooperation among crop physiology, crop agronomy

and climate science would be a positive outcome of the Royal Society meeting.

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No royal road to protein structure determination

SIR — Your News story "Protein structures hint at the shape of things to come" (*Nature* **435**, 547; 2005) projects an excessively optimistic view of structural genomics, which your readership might find misleading.

The claim that "structural genomics initiatives are ready to roll out protein structures in large numbers" should be treated with scepticism. An alternative view predicts that the number of protein structure determinations will peak and then decline, as the pool of easy targets (so-called 'low-hanging fruit') gets depleted.

Recent technological advances have considerably eased the transit through some of the bottlenecks of crystallographic analysis. However, the unique biochemical properties of individual proteins continue to present a formidable barrier to any automated procedure of structure determination. A better way to assess the outcome of high-throughput initiatives would be to consider their overall success rate, given as the ratio of structures solved to the number of targets attempted.

It is for the grant agencies to decide whether funding of structural genomics programmes constitutes a worthwhile investment. Perhaps the most serious point, though, is that the wider scientific community might come to consider all structural biology endeavours as straightforward. This is not the case, especially at the frontier of modern structural biology, where the crystallographic analysis of large macromolecular complexes still requires heroic efforts. In this respect, the image that accompanies your News story is of a protein that structural biologists would consider not particularly challenging, and that is simpler than the four protein structures reported elsewhere in the same issue, obtained through traditional structural-biology approaches.

When asked by King Ptolemy I about an easy way to learn geometry, the Greek mathematician Euclid famously replied that there was no royal road to geometry. Paraphrasing Euclid, we could affirm with equal confidence that there is no royal road to protein structure determination.

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COMMENTARY

When will we tame the oceans?

In fisheries across the world, fish stocks are declining fast. Future preservation and management of the ocean's resources will require a transformation of our relationship with the seas, argues **John Marra**.

Fishing in the ocean is no longer sustainable. Worldwide, we have failed to manage the ocean's fisheries — in a few decades, there may be no fisheries left to manage¹. So what should be done?

Following the cultivation of land for food, society must take the next step: largescale domestication of the ocean. Last month, the US National Oceanographic and Atmospheric Administration proposed legislation to expand fish farming in US federal waters up to 200 miles from the coast, and to increase the numbers of species that can be farmed. Many reacted with dismay to this announcement². But I believe these people are ignoring the inevitable.

Aquaculture is entirely responsible for the increase in world fish harvests that has occurred in the past 18 years³. We have already accepted domestication of the land; now is the time to accept the same for the seas. The land was transformed with little consideration for the consequences. For the ocean, we will have to decide, and soon, how domestication should take place, so that it is managed in ways that maintain environmental health and sustainability.

Since the end of the last ice age, and increasingly since the beginning of the industrial revolution, humans have affected the evolution of plant species, found uses for animals, and caused losses of wildlife⁴. In short, we have altered land-based ecosystems to serve our own ends, reaping the benefits (civilization) and also bearing some of the costs (for example, increases in animal-borne disease). Similar changes have occurred in the ocean, albeit more slowly because of its size and inhospitable nature. So far, changes in the ocean mirror what has happened on land: habitat destruction, major shifts in the communities of plants and animals, and the loss of larger animal species.

Fishing, which is essentially hunting in the ocean, is a direct or indirect cause of many of these changes — from the loss of large marine mammals to habitat destruction. Incessant hunting, with increasing technological profi-



KONA BLUE WATER FARMS

CATES INTERNATIONAL



Hemmed in: giant offshore fish pens are currently being developed as a means to harness the sea's resources.

ciency, has decimated fish populations worldwide. Catches of large marine species, such as swordfish and tuna, have declined by 80% over the past 20 years⁵. Northern cod, historically a dietary mainstay, and a species once thought inexhaustible, is all but commercially extinct in the western North Atlantic. In many areas, bottom trawls have scoured the seabed clean. These are just a few examples of the "long and miserable record" of hunting in the ocean⁶.

If history is a guide, the ability of the ocean to supply the fish we take will soon reach its limit despite the best efforts at management, such as environmental restoration⁷ or ecosystem-based fisheries management⁸. The collapse of ocean fisheries comes at a time of accelerating demand for food, especially of animal protein. World food production must double in the next 50 years to keep pace with

population growth⁹, and the world's oceans must play an increasingly important role as a food source. In the past 20 years alone, farming of marine fish and shellfish along coastlines has grown about 10% per year¹⁰.

So far, most of the expansion in aquaculture has come from farming freshwater species, such as tilapia and catfish, in ponds. But marine aquaculture, or mariculture, is also growing, and given eventual limits to space on land, moves towards expanded mariculture, such as the recent US proposal, will accelerate in the years to come. The demise of ocean fisheries and the destruction of marine habitats help to explain why ocean domestication is inevitable. But we need to carefully consider how this domestication should happen to avoid many of the pitfalls cited by environmentalists and scientists¹⁻³.

As with the rearing of land animals, fish farming can harm the environment in many ways; indeed, some mariculture operations have caused whole-scale destruction of coastal ecosystems¹. First, marine farming can pollute in ways that are aesthetically, chemically and genetically destructive. Coastal mariculture systems spoil ocean views and affect property values. Chemicals added to fish feed,

such as colourings and hormones, find their way into the seabed, from where they can enter benthic food webs. And genetic pollution, whereby domesticated species escape their enclosures and infect the gene pool of wild fish stocks or replace endemic species, is a troubling prospect. Second, crowding in aquaculture enclosures or ponds can easily amplify disease and cause it to spread more quickly than it would in the wild. Third, the mariculture of carnivorous species puts additional pressures on fisheries to provide ever-larger quantities of wild fish for feed³, exacerbating the decline of wild fish populations. In some fish farms, the consumption of small pelagics, such as anchovy or sardine, by commercial fish (salmon, for example), actually exceeds commercial-fish production, in terms of biomass.

There is no question that these are significant problems to overcome; such environmental ills echo those visited on the land since the advent of farming. But if we recognize that domestication of the ocean is starting to happen, we can craft a research agenda to mitigate the problems and maintain both economic and ecological sustainability.

Research questions for the mariculture industry span basic and applied research, and policy — from marine biology and physical oceanography, to engineering and the law. Which fish species can be adapted to captivity, that is, domesticated throughout their life cycle? How can their health and diets be maintained? Are there alternatives to the small pelagics currently fed to farmed fish, such as by-catch? Where should mariculture systems be situated, in terms of ocean dynamics and the surface wave environment? How should they be constructed and maintained? Answering these questions in turn raises legal and policy concerns.

One solution to many of the problems associated with coastal mariculture is to move the systems further offshore — to the waters of the outer continental shelves, and beyond to the open ocean. Generally, offshore systems cause less coastal pollution, but can dramatically increase costs. Nevertheless, pilot projects under development illustrate the potential for creative solutions.

For example, giant versions of coastal fish pens (containing about 100,000 m³ of water) are being designed to drift freely in the ocean¹¹. Prototypes, shaped as two cones sandwiched together at their base, are currently being tested in some coastal sites, while remaining tethered to the sea floor. Under certain proposals¹², fingerling fish will eventually be placed in pens in Florida. Then, the Gulf Stream and North Atlantic currents will carry the pens with their contained fish across the Atlantic, feeding them along the way. Designed to drift at depths well below the surface, the pens' interference with shipping will be minimal. Arriving in Europe many months later, the fish will have grown to a marketable

size. After harvesting, the pens can be reloaded with fingerlings for a return voyage. But such systems represent a significant engineering challenge: feeding, maintenance of an ideal depth and communication via satellite must all happen automatically, for months at a time.

Another development concerns tuna 'herding' at sea. Many species of tuna are attracted to anything that is sufficiently different from their surroundings. Fishermen take advantage of this behaviour by using a fish aggregating device or FAD. This can be as simple as a floating log, or a more complicated buoy system, or even a disturbance at the ocean surface. Fishing boats directing a fire hose behind them create just such a disturbance, and can thereby attract a following of tuna. The tuna attracted to FADs can be fed, maintained, and a portion eventually harvested¹³. Although their use is controversial¹⁴, FADs could increase yields. Tuna will never be domesticated, as are sheep or cattle, but the analogy of herding on the 'high plains' of the sea still holds.

These are only two examples of how we might enhance ocean fish yields without further damaging ocean ecosystems through fishing, and solve the problems caused by mariculture near coastlines. But we need to be clear about the wider consequences of domesticating the ocean: first, like the hunters and trappers of the American west 150 years ago, fishermen will largely disappear; game fish will be taken by a regulated number of recreational fishers; and the bulk of the fish we eat will come from more limited varieties. In such a future, we will have to accept an ocean with fish that can be cultured, and we will have to accept less freedom of the seas.

Internationally, domestication will entail a reordering of the status quo. Today, govern-

ments and fishermen have become accomplices in the over-exploitation of fish stocks and over-capitalization of the fishing industry. Instead of competing for a rapidly dwindling resource, maritime nations will have to negotiate agreements concerning the shared use of the ocean. Governments also have a role to play in fostering research to find ways to cultivate fish in the open sea, and more environmentally sound ways to conduct mariculture near shore. The common goal should be to maintain the ocean as a sustainable source of food, both economically and ecologically. As on the land, sustainability of the ocean's food supply for the world's population means domestication of the seas. ■

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Acknowledgements I thank G. Pontecorvo, H. J. Simpson, H. Bates, J. G. Shepard, J. Steele, N. Marra, M. Tobin and D. Bates for comments.

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

Pollution caused by fish farming in coastal waters could be reduced by moving the farms further offshore.

BOOKS & ARTS

The Arctic paradox

The traditional diet of Inuits has health benefits but exposes them to dangerous levels of pollutants.

IMAGE
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BRYAN & CHERRY ALEXANDER PHOTOGRAPHY

Silent Snow: The Slow Poisoning of the Arctic

by Marla Cone

Grove Press: 2005. 256pp. \$24

Geir Wing Gabrielsen

In 1962, Rachel Carson published her famous book *Silent Spring* about man-made chemicals found in the tissues of wildlife and humans. In so doing she started a debate in the United States and Europe about the production of chemical pollutants. Partly in response, the environmental movement grew and the public demanded that government and industry be held accountable for their actions.

Several decades later, Dianne Dumanoski, Theo Colborn and John Peterson Myers published *Our Stolen Future* (E. P. Dutton, 1996), which demonstrated the effects of man-made chemicals on wildlife. The book launched some major research initiatives to investigate the effects of chemicals (such as endocrine disruptors) on humans. Now, nearly ten years later, Marla Cone presents *Silent Snow*, a comprehensive book about pollutants found in the Arctic ecosystem and their effects on both wildlife and people.

The Arctic is generally considered to be pristine, as there are few local sources of pollution. However, during the past 10–15 years there has been increasing evidence (mainly from the Arctic Monitoring and Assessment Programme) that the Arctic environment is threatened by pollution and climate change. Cone, an environmental reporter at the *Los Angeles Times*, describes the 'Arctic paradox', in which people living in the Arctic are the most contaminated, despite the fact that they live far away from the sources of these pollutants. She describes how the Inuit in Canada and Greenland face a significant health risk as a result of their dependence on traditional food — meat and blubber from seals and whales — that contains high concentrations of pesticides and industrial pollutants.

Because of long-range transport by air, water currents and river outflow, the Arctic is a sink for industrial and agricultural pollutants from the south. When these accumulate in Arctic marine food webs, they affect humans and wildlife. In some human populations living in the Arctic, levels of persistent organic pollutants (POPs) and mercury are the highest anywhere on Earth and exceed health and safety guide-

lines. In children living in northern Canada, POPs have been documented to affect the immune system, as shown by higher than normal rates of infectious diseases. On the Faroe Islands, which were also visited by Cone, high mercury levels cause irreversible neurological damage to fetuses, leading to reduced learning ability later in life. As a result of these findings, children and women of reproductive age in some Arctic populations are being advised to reduce their intake of traditional foods.

Silent Snow is a journey into the science of toxicology and the ecological impact of pollutants on animals and people living in the Arctic. Cone has visited scientists in the field and in their labs and presents a fascinating but tragic story about the problems of Arctic pollution. She also talked to native people in Alaska, Greenland and the Faroe Islands, and illustrates the problems these people face if they continue their way of life and dependence on traditional food. They eat a marine diet, considered to be among the world's healthiest (based on the content of iron, proteins, vitamins and omega-3 fatty acids) — but the levels of pollutants are so high that it threatens their wellbeing. Unfortunately, switching to Western

food increases the risk of other diseases not normally found in these populations, such as cancer, heart disease and diabetes. This poses a dilemma for public-health officials: they encourage the Inuit and others to eat traditional foods, but advise them to reduce their consumption of such foods.

I enjoyed reading Cone's book, especially her descriptions of conversations with indigenous people and scientists in different parts of the Arctic. This discussion would have been improved if she had also visited the indigenous families of the Russian Arctic. These families, which constitute almost half of all indigenous people in the Arctic, face serious health risks. The collapse of the Soviet economy left them dependent on traditional food, increasing their risk of chemical exposure.

Cone presents the science of Arctic toxicology and the Arctic paradox in an interesting and readable way. She also presents some solutions and predictions about the pollution problem. The Stockholm Convention, which

will ban the use and production of a 'dirty dozen' chemicals, is an important step towards a reduction in the production and use of man-made chemicals. Although the concentrations of some chemicals (such as PCBs and DDT) are decreasing in the Arctic environment, others are becoming more common, especially mercury, brominated and fluorinated compounds. There is an urgent need for action, from both industry and government agencies. To this end the European Commission has made a plan for the testing and regulation of chemicals. Unfortunately, it seems harder than ever to ban toxic substances in the United States.

Silent Snow is an important book that should be read by environmentalists, scientists, politicians and the public. The environmental problem of man-made chemicals, addressed in this and previous books, should send a clear message to the rest of the world. ■ Geir Wing Gabrielsen is at the Norwegian Polar Institute, Polar Environmental Centre, Hjalmar Johansensgt 14, 9296 Tromsø, Norway.

that people possess free will and are responsible for their actions with the scientific view that, as physical objects, our actions are fully determined. Gazzaniga's solution is to distinguish brains from people — "Brains are automatic, but people are free." Responsibility is "a social construct that exists in the rules of a society, [it] does not exist in the neuronal structures of the brain". For him, scientists have nothing to say about such issues: they should stay in their labs and out of the courthouse and legislature.

This may be a bit too cautious. Even if Gazzaniga is right that responsibility is a social construct and that for a neuroscientist, no person is more or less responsible than any other, there are reasonable and unreasonable ways to apply this construct. If a paranoid schizophrenic kills someone while in a delusional state, we do not (and should not) punish him or her as we would a mafia hit man, because of what we know about schizophrenia. In this regard, science does bear on questions of moral responsibility, particularly with regard to difficult issues such as how to deal with crimes committed by teenagers, or by those with learning difficulties.

Gazzaniga is a lot less cautious when it comes to the implications of neuroscience for ethics in general. As he puts it in his preface, "I would like to support the idea that there could be a universal set of biological responses to moral dilemmas, a sort of ethics, built into our brains. My hope is that we soon may be able to uncover these ethics, identify them, and begin to live more fully by them. I believe we live by them largely unconsciously now, but

Dissecting the right brain

The Ethical Brain

by Michael S. Gazzaniga

Dana Press: 2005. 226 pp. £17.50, \$25.00

Paul Bloom

It matters to me what Michael Gazzaniga thinks about the brain and, if you live in the United States, it should matter to you too. In 2002, Gazzaniga was appointed to the President's Council on Bioethics and so his views on cloning, euthanasia, neurological enhancement and embryonic stem cell research will help shape US law and policy. Gazzaniga is an admirably clear writer who assumes no expertise on the part of his reader. Although he says that *The Ethical Brain* was written to encourage fellow neuroscientists to enter the public debate on these issues, it could be read by anyone who has an interest in the controversies that lie at the intersection of science and ethics.

Gazzaniga's main point can be summarized as: Don't Panic. He is sceptical that we will ever be able to create 'designer babies' or pills that lead to effortless improvements in human performance. He argues that 'mind-reading' techniques such as functional MRI and implicit tests of racial bias are actually of limited value when it comes to determining moral or legal responsibility. And he is confident that individuals can make competent decisions about the proper use of technologies such as cloning and neurological enhancement. With just a few exceptions, he believes the government should stay out of such decisions.

He is particularly dubious about slippery-slope arguments of the type: we can't let people

do X, because even though X is ok, it might lead to Y and Y is terrible. As he puts it, "It does not make moral, political, or social sense to allow the fear of the extreme to hinder the good."

He also addresses the big questions, such as how to reconcile the common-sense notion

IMAGE
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Left hanging: this demonstration in April 2002 put pressure on the US Senate not to ban therapeutic cloning.

that a lot of suffering, war, and conflict could be eliminated if we could agree to live by them more consciously."

This conclusion would follow if our universal moral sense had been implanted by an all-knowing and all-loving God. But biological evolution is a notoriously amoral force. Innate moral universals would have been shaped by the selective advantages that arise from caring for our kin and cooperating with our neigh-

bours, but nothing in our genes tells us that slavery is wrong, or that men and women deserve equal rights. Such insights emerge through individual and group processes that engage all of our faculties, including our innate moral sense, but also the capacity to appreciate abstract arguments, formulate analogies, learn from experience, take other's perspectives and so on. Much of moral progress consists of using reason to override our gut feelings.

An excellent illustration of why ethics does not reduce to instinct comes from Gazzaniga's own treatment of issues such as stem cell research and euthanasia. This shows ethical reasoning at its best — rooted in common sense but also informed by a sharp, inquisitive mind and a deep appreciation of the facts. ■ Paul Bloom is in the Department of Psychology, Yale University, 2 Hillhouse Avenue, New Haven, Connecticut 06520-8205, USA.

Eddies at The Gates

An art installation hints that, even in a forest, wind may disperse tree seeds farther than expected.

Henry S. Horn

Tree seeds that are dispersed by the wind have parachutes, wings or sails to slow their descent. This keeps them in the airflow longer, allowing them to travel farther. But the assumption has been that a seed falling in a forest is doomed to travel only a short distance, because the wind is impeded as it passes among the trees.

To travel far, a seed must rise above the forest canopy on an updraft whose velocity exceeds the rate of fall of the seed in still air. If the updraft is part of a coherent rolling eddy, the seed might 'surf the wave' to a great distance. To guess how far a seed might get, it becomes important to know the sizes and lifespans of coherent wind eddies, but it seems that no one has made the appropriate measurements.

My colleagues and I have produced computer models that predict wind dispersal over long distances, but it has been difficult to convince others that our models are realistic (Nathan *et al.* *Nature* **418**, 409–413; 2002; *Div. Distrib.* **11**, 131–137; 2005). A visit to The Gates, Christo and Jeanne-Claude's temporary art installation in New York's Central Park this February allowed me not only to visualize coherent eddies, but also to measure their sizes and local lifespans. The measurements came from 57 photographs that I took with a digital camera on the afternoons of 24, 25 and 27 February 2005.

The Gates in Central Park, New York City, 1979–2005 comprised 7,500 gates, around 4 metres apart and 5 metres high, following the line of the paths through the park. Saffron-coloured fabric panels were hung from the top of each.

When there was no detectable wind or only a light breeze, the fabric of The Gates hung vertically with minimal flutter. The panels billowed out to within 20° of the horizontal for winds recorded near the ground at 2 to 5 metres per second (www.cdo.ncdc.noaa.gov/ulcd/ULCD). I measured the 'footprint' of a coherent eddy by counting a consistent number of



Seeing ghosts: a 58-second sequence showing the waxing and waning of a 25-metre eddy.

contiguous billowing gates and calculating the distance they span. Footprints spanning at least 12–13 gates (about 45 metres) were common at wind speeds of 2 to 5 metres per second. From timed sequences of photographs of sets of gates, I recorded local lifespans of 32 to 57 seconds.

These records are biased toward shorter lifespans, as I chose to photograph sequences only when the gates were changing orientation rapidly. Some eddies lasted longer than 100 seconds, and the their footprints tended to move along a line of gates at scales of about 100 metres.

Seeds that are kept aloft by updrafts in a 45-metre coherent eddy, for 50 seconds, in a horizontal wind of 5 metres per second, could travel at least 0.25 kilometres. This is farther than we thought, even though the measurements behind the calculation are all substantial underestimates.

Thanks to Christo, Jeanne-Claude and The Gates, I now have direct quantitative observations, in moderate winds, of the

coherent eddies that are crucial to long-distance dispersal of seeds and other biotic agents. Praise is also due to Christo for having anticipated realistic sizes for the wind eddies that drive The Gates in his conceptual drawings, which were made before construction of the work itself.

Of course, the aerodynamic presence of The Gates is part of the landscape that may interact with the generation and propagation of coherent eddies. Some details are likely to be peculiar to Central Park, and even to the installation itself. Nevertheless, the general pattern and its spatial and temporal scales are highly suggestive of features to be expected in a natural landscape. So the seed can indeed fall far from the tree.

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DEVELOPMENTAL BIOLOGY

Tiny brakes for a growing heart

Benoit G. Bruneau

The discovery of microRNAs has revolutionized many areas of biology. The latest news is that these RNAs seem to regulate the crucial balance between growth and specialization of cardiac cells.

MicroRNAs (miRNAs for short) are tiny RNA molecules that downregulate protein production, either by inhibiting the translation of protein from messenger RNA or by promoting the degradation of mRNA^{1,2}. In plants, miRNAs have major influences on embryonic development, primarily by slowing down the production of proteins that regulate gene expression. In vertebrates, the functions of miRNAs are less apparent, but — intriguingly — some miRNAs are expressed in specific tissues^{3–6}. How these miRNAs are restricted to, say, the heart or the brain is not known, nor is it clear what they are doing there. In this issue, striking results from Zhao *et al.*⁷ (page 214) implicate miRNAs in controlling heart development in mouse embryos. The work places miRNAs both upstream and downstream of known cardiac gene-regulation cascades, and provides an exciting basis for understanding the regulation of organ formation.

Zhao *et al.*⁷ characterized two related miRNA genes, *miR-1-1* and *miR-1-2* (collectively known as *miR-1*), the products of which are enriched in heart and muscle^{3,4}. Locating miRNAs in embryonic structures is technically challenging. To achieve this, Zhao *et al.* used a ‘reporter’ that produces a blue stain, which means it can easily be tracked. To mimic the expression pattern of the *miR-1* gene, they spliced the gene encoding the reporter to the stretch of DNA in front of the *miR-1* gene — the equivalent region of a protein-coding gene would normally carry regulatory information.

Zhao *et al.* found that the reporter was expressed in the heart and muscle of embryonic mice, and that the expression was controlled by several key heart and muscle transcription factors (proteins that regulate gene expression): serum response factor (SRF), Mef2 and MyoD. These results show that miRNAs are regulated just like ordinary genes, and therefore can potentially respond to the same developmental cues as other genes. As with miRNAs in zebrafish⁸, mouse *miR-1* switches on rather late in heart development, implying that it acts after some of the main developmental steps have taken place.

So what does *miR-1* do in the heart? Some clues about the function of miRNAs in embryo

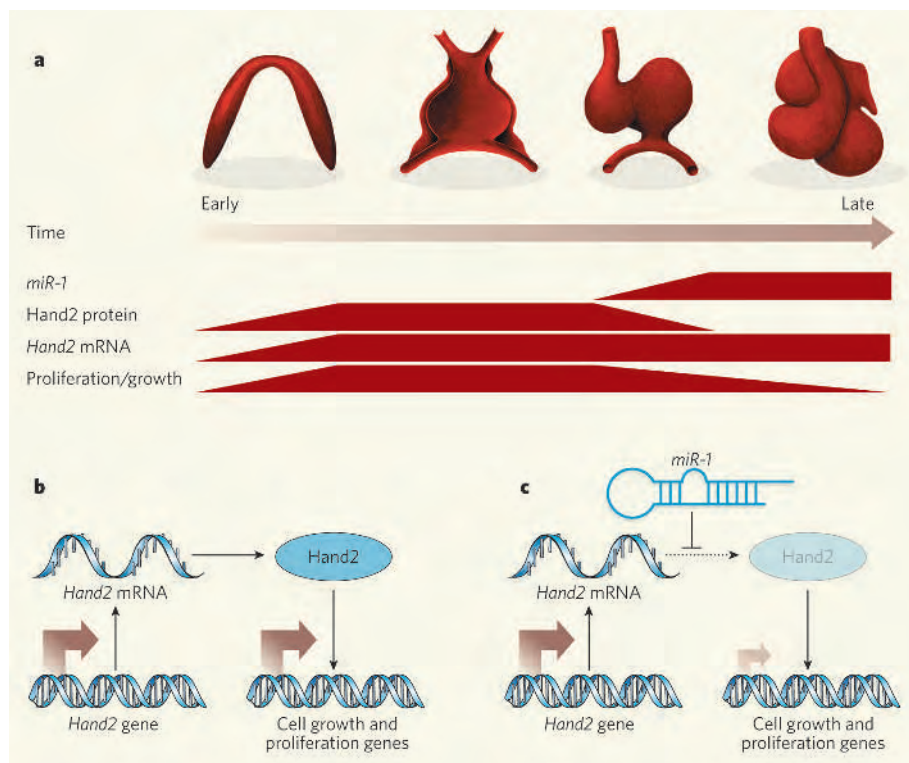


Figure 1 | Heart development and *miR-1*. **a**, The timing of *miR-1* action during heart development. In the early stages of heart development, the gene encoding *miR-1* is off, and Hand2 is produced. At later stages, *miR-1* switches on to reduce production of Hand2 protein, thereby slowing the growth of the heart. **b**, **c**, Proposed mechanism of *miR-1* function. **b**, The expression of the *Hand2* gene produces an mRNA that is normally translated into Hand2 protein. **c**, After Hand2 has completed most of its developmental tasks, *miR-1* halts the translation of the protein.

development come from experiments that disrupt the formation of all miRNAs by inactivating Dicer, an enzyme essential for miRNA production. In zebrafish, inactivation of Dicer leads to defects in the formation of the brain and heart⁸. However, it is not clear which miRNAs have developmental roles, or which genes they target. Using mice that are genetically modified to express *miR-1* throughout the heart from a very early stage in heart development, Zhao *et al.* show that precocious expression of *miR-1* leads to defects in heart formation because of decreased cell division (hence fewer heart cells). So, *miR-1* may help regulate the balance between cardiac differentiation (specialization) and cell division, by

slowing down proliferation at the right time.

But what are the targets of *miR-1* in heart development? A major appeal of Zhao *et al.*'s work⁷ is that the authors claim to have devised a better way of identifying ‘real’ miRNA targets. Several approaches have been published, each claiming to be more efficient and specific than the previous one, so what makes Zhao *et al.*'s approach stand out? Significantly, their predictions are borne out by real data from an experimental system. However, they seem not to have found all the *miR-1* targets, so although their predictions are correct, the criteria used by Zhao *et al.* may have been too restrictive to pick out all the targets. Time will tell if their method is indeed superior.

One of the principal *miR-1* targets they identify is Hand2, a cardiac transcription factor (Fig. 1). Hand2 is expressed early in heart development and is involved in regulating its growth⁹. However, Hand2 is expressed continuously throughout heart development, and it is not known whether it continues to perform the same functions late in development as it did during the earliest stages. A simple interpretation of these observations would be that after Hand2 has performed its crucial tasks, *miR-1* halts the production of Hand2 protein from the *Hand2* mRNA, thus slowing down the growth of the heart (Fig. 1b, c).

Of course one might ask, why not just turn off the *Hand2* gene at the right time? Because miRNAs have several targets, it may be more efficient to slow the production of several proteins by simply turning on one miRNA gene. Therefore the role of developmentally expressed miRNAs may be to titrate out regulatory molecules, such as transcription factors, to finely regulate organ growth and differentiation.

Zhao *et al.*'s findings open up a whole new dimension to the regulation of organ formation, by adding an miRNA-dependent regulatory step to the complex role of transcription factors. But many questions remain. For example, how are *miR-1* genes turned on so

relatively late in development? Are there factors that delay the activation of the *miR-1* genes, and if so what are they? Moreover, it is unlikely that *Hand2* is the sole target of *miR-1* in the heart, so what else needs to be halted by *miR-1* for normal heart development to proceed? The genetic deletion of *miR-1* will no doubt help to answer these questions. Finally, because aberrant cardiac patterning during embryonic development can lead to congenital heart defects in humans^{9,10}, are miRNAs also involved in these disease processes? ■

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ASTRONOMY

Giant planet seeks nursery place

Artie P. Hatzes and Günther Wuchterl

A further discovery of a planet in a binary star system — this time close in — could prove a problem for accepted theories of planetary formation. The implication is that there are more planets out there than we thought.

The first discovery ten years ago of 'Pegasi' planets¹ — giant planets 100 times closer to their star than the Solar System's giant planets are to the Sun — shook long-held conceptions of planetary-system formation, which took the Solar System as a template. To square the new observations with the conventional model, some astronomers adopted theories of violent planetary migrations, in which the planets move around after they form. The discovery by Maciej Konacki (page 230 of this issue)² of a giant planet in a system where the gravitational pull of a second star would disturb the planet's putative nursery will now place severe constraints on such theories.

Astrophysicists largely agree that giant planets form from a protoplanetary disk that surrounds an infant star — exactly how is a subject of debate. Before the discovery of extrasolar planets (planets beyond our Solar System), Jupiter was assumed to have grown from a core of solid material that, once its gravitational pull was great enough, attracted gas (mostly hydrogen and helium) until it

reached its current size of 318 Earth masses. Such 'core accretion' only occurs if there is enough solid material to start with — this would preferentially be in cool regions of the protoplanetary disk far from the star, where the heavy elements are largely in a condensed form. The so-called ice line, beyond which this occurs, is typically at least 3 AU from the star. (1 AU, or astronomical unit, is the Earth–Sun distance.)

Thus, the discovery of 51 Peg¹, a giant gaseous planet that orbits its parent star at only 0.05 AU, was problematic: if this planet had to have formed at a distance of at least 3 AU, what was it doing so close to its host star? Orbital migration, introduced by theorists to explain this discrepancy, holds that, once the star had accreted a critical mass of around one Jupiter mass, gravitational forces between it and the protoplanetary disk caused the planet to lose angular momentum and thus spiral towards the star. (How Jupiter remained at its original distance is unclear in this theory.) Competing theories of *in situ* formation of 51 Peg have

been proposed^{3,4}, but the inner regions of the protoplanetary disk have only enough material to form planets with sizes totalling a few Earth masses — too little to attract the hydrogen and helium gas needed to form a giant planet. Thus, the model of core accretion and ensuing orbital migration became widely accepted as explaining the formation of giant planets.

This model is challenged by Konacki's discovery², which was made with the Keck Telescope on Hawaii. Konacki used the 'Doppler wobble', a now-standard technique that measures the subtle acceleration of a host star that results from the presence of an unseen companion. What Konacki found is another giant planet with a short orbital period (more than 30 are known). The importance of the finding lies in where he found it — in orbit around a star that is part of a close binary system called HD 188753.

More than 60% of stars in our Galaxy are gravitationally bound to a companion, so the Sun is in a minority. Most searches for extrasolar planets have avoided such binary systems because light from two stars complicates analysis, and it is not clear how the presence of a second star would disturb the process of planet formation. Planets have been discovered in binary systems, but only where the second star is too far away from the first for it to have much influence — the closest binary system where a planet had been discovered was the brighter component to γ Cephei⁵, where the orbital period of the two stars round each other is some 56 years.

The binary orbital period of HD 188753 is just 25.7 years, and the orbital separation of the stars, both of Sun size, is a mere 12.3 AU — about the distance from the Sun to Saturn. Konacki's velocity measurements reveal that the primary star (the more massive star, denoted HD 188753A) has a planetary companion of a minimum of 1.14 Jupiter masses that orbits the star every 3.35 days at a distance of about 0.05 AU. Yet according to the orbital migration theory, this planet should not exist. The secondary star is so close that its gravitational pull would have stripped away the protoplanetary disk of the primary star — where, even if it later migrated, the planet must have formed — reducing the disk to a radius of just 1.3 AU. But within this radius, ices are unlikely to last and so cannot contribute to the formation of a massive core. The alternative explanation — that the planet formed where it is — would challenge the standard picture, but runs into the problem of where the necessary solid material came from.

Further evidence⁶ that the region in which a giant planet can form might be smaller than current models allow comes from the exoplanet host star Gl 86, whose companion star, thought to be of brown-dwarf mass, has now been shown to be a white dwarf — an exhausted giant star that has collapsed in on itself. Depending on how big this second star was, there may also have been no ice-containing

outer region of the protoplanetary disk in this system.

Although the planet in the HD 188753 system² presents a conundrum to theorists, there might be an easy way out: abandon the make-do-and-mend migration theory to Occam's razor, and accept that not all planet-forming nebulae are similar to the solar nebula. Large and small protoplanetary nebulae of the same mass might differ only in their total angular momentum, such that in smaller nebulae more mass is closer in — nursing young giants.

The giant planets that orbit other stars exist in a diversity of systems and most are unlike the system of planets found around our own Sun. In our view, the diversity of planetary systems probably reflects a diversity of protoplanetary nebulae, and wherever sufficient mass is available, planets, even giant ones, may form. The neglected majority of double stars could thus fill the Galaxy with planets.

Sometimes we stumble across a planet in a place we did not expect to find one. Such a discovery can provide important steps towards understanding just how our Solar System, and others, formed. The discovery of the HD 188753 planet may be one of those steps. ■

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EVOLUTIONARY BIOLOGY

Relativity for molecular clocks

David Penny

An analysis of genetic data sets from primates and birds provides firm evidence that molecular evolution is faster on shorter than on longer timescales. The estimated times of various evolutionary events require a rethink.

The relative constancy of the rate at which DNA sequences evolve has been a treasured icon of molecular evolution for nearly 40 years. The occurrence of such a stochastic 'molecular clock' was initially quite unexpected, and was explained by Motoo Kimura¹ by assuming that most changes to amino-acid and nucleotide sequences were neutral — "neither beneficial nor injurious", in Charles Darwin's prescient phrase.

However, there have been several inklings^{2–4} that the rate of molecular evolution accelerates when measured over evolutionarily short timescales. As they report in *Molecular Biology and Evolution*, Ho and colleagues⁵ have now put the evidence together. Their analyses of primate and bird data sets reveal that there is indeed a decided acceleration of molecular evolution on short timescales. This is an effect that demands explanation; moreover, estimates for the timing of recent events in population biology will need to be reconsidered.

First, some background. Traditionally⁶, the total mutation rate μ is subdivided into the proportion of mutations that are advantageous (α^+), neutral (α^0) or deleterious. Most rate calculations focus on neutral and deleterious mutations. Kimura's argument¹ for the long-term rate is that for a fixed population size of N individuals, with a single copy of their genome (haploid), each neutral variant has the same probability of $1/N$ of ending up in 100% of the

population (that is, becoming fixed). Kimura's basic argument is that the number of neutral mutations per unit time is $N\mu\alpha^0$, and because the probability of fixation of a single mutation is $1/N$, the rate of fixation is $\mu\alpha^0$. If the rate of fixation is equated with the rate of long-term molecular evolution, they will both equal the rate of neutral mutation, $\mu\alpha^0$.

Here I'll consider four questions. Is the short-term acceleration of molecular evolution real? If so, is it explainable? If so, why wasn't it detected earlier? Finally, what are the consequences?

First, is the acceleration real? Ho *et al.*⁵ observed the effect in three data sets, two based on protein-coding genes (avian and primate) and the third on the control regions in the mitochondria of higher primates. In each case, at times less than about 1–2 million years there is an increasing acceleration, with the highest rates at the shortest times. The rate is highest between generations (that is, in pedigree studies), and decreases continuously for local and then for widespread populations. Finally it reaches the low plateau that we know for long-term evolution. Overall, there is the J-shaped rate curve shown as the solid line in Figure 1a — in Texas-talk, the lazy J.

The authors⁵ eliminate both sequencing and calibration errors, as well as saturation of mutations at fast-evolving sites, as explaining the change in rates — although calibration errors at the shorter times (within the past million years) contribute strongly to the variance.

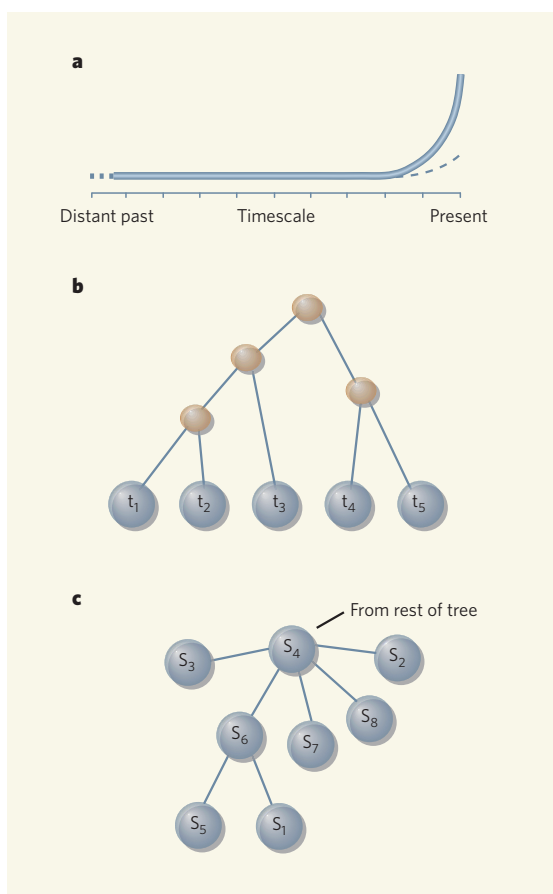


Figure 1 | The J-shaped rate curve and the multiscale problem. **a**, The shape of the rate curve of molecular evolution as estimated by Ho *et al.*⁵. The higher rate at shorter times is interpreted here as having a neutral component (below the dashed line) combined with an effect from deleterious mutations. **b**, In the classic long-term problem in evolutionary biology, each taxon is represented by a single sequence ($t_1, t_2, \dots$) and a tip-labelled binary tree is appropriate; the internal points are 'missing data'. **c**, Within populations there will be many sequences ($s_1, s_2, \dots$) per population. At these shorter times the tree is usually multifurcating (not binary), and sequences occur at internal nodes (representing ancestral nodes still present in the population). Comparison of **b** and **c** shows that there is a multiscale problem, where different aspects of the same underlying process are observed as the scale varies; in this case, the scale is time.

Overall, however, the implication is that the timing of many recent events in human evolution has been overestimated by past studies. Examples include the divergence between humans and Neanderthals — new estimate 354,000 years ago (range 222,000 to 705,000 years ago, against a current range of 317,000 to 853,000 years ago); and the last split within those Neanderthals that have been sequenced — 108,000 years ago (range 70,000 to 156,000 years ago compared with a current range of 151,000 to 352,000 years ago).

Second, can the acceleration be explained? One approach is as follows. We can subdivide the deleterious mutations into the proportions that are very slightly deleterious, α^- , slightly deleterious, $\alpha^=$, and deleterious (but not lethal), α^+ . Deleterious mutations are not expected to become fixed in large populations, but nevertheless can persist in the population for long periods of time. The average time before loss (see ref. 7, for example) correlates with deleteriousness, so persistence time increases from $\alpha^=$ to α^+ . Thus, as observation times diminish, we should observe a greater proportion of slightly deleterious mutations that have yet to be lost, with the most deleterious (α^+) observed only in the short-term pedigree studies. This gives the apparent acceleration in mutation rate as the separation times between sequences decrease. A qualitative explanation such as this is straightforward, but the effect requires quantitative treatment and thorough testing.

Third, why wasn't the short-term acceleration picked up earlier? There were hints. Kimura¹ (page 45ff.) comments that his calculations assumed sufficient time for loss or fixation of a neutral mutant, and that at shorter intervals there would be higher variability. Some of the increase in rate comes from neutral mutations (Fig. 1a), and some from slightly deleterious mutations. In early molecular work, the genetic diversity within populations — heterozygosity — was measured by the differences in the behaviour of variant proteins in electrophoresis, and long-term evolution by differences in amino-acid sequences between species (with only one sequence studied per species). Thus, direct comparability was not possible in those early studies — although in 1977 Ohta did point out⁸ that "In the future, it is likely that genetic variability will be detected at the level of amino acid or nucleotide sequences".

For some reason, the continuum between population heterozygosity and long-term evolution has not been adequately studied. Although it is a continuum, the techniques required may change as the timescale decreases. For example, some concepts from long-term evolution (binary evolutionary trees with sequences studied only at the tips) have been extended into populations where trees are no longer binary, and ancestral sequences (at internal nodes) are still present in the population. There are hints that a formal multiscale study⁹ is necessary, because even

though the same underlying process is occurring, different features of trees are observed as the timescale changes (Fig. 1b, c).

Finally, what are the consequences of Ho and colleagues' conclusion⁵? As they point out, the obvious ones are practical, in that many time estimates require recalculation — including the times of events in recent human evolution (such as origin of the Polynesian genetic marker that distinguishes Polynesians from all other human populations¹⁰), and the origin of RNA viruses such as HIV¹¹. In some cases the constraints are from recent events, and it is the long-term events that require re-analysis.

Much more remains to be done. There is the challenge of formulating a single theory that operates smoothly over disparate timescales, from current heterozygosity to the long-term rate of evolution. In addition, a single mutation rate (μ) does not really exist. Even for nucleotides there are many 'mutation rates', at least one between each pair of nucleotides, and these can be estimated separately using three-

dimensional matrices¹². The J-shaped curve cannot rest until a single theory holds for it: we live in interesting times. ■

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CANCER BIOLOGY

Sleeping Beauty awakens

Keith C. Weiser and Monica J. Justice

Ancient jumping DNA found napping in fish has been revived and is being used to identify cancer genes in mice. But the benefits of this aptly named 'Sleeping Beauty' system could reach far beyond cancer.

Transposable elements are discrete pieces of DNA that can jump around in the genome of a living organism. These elements were initially discovered in corn through the Nobel-prize-winning work of Barbara McClintock¹, and they have been found in organisms throughout the tree of life. For each DNA transposon, a corresponding protein, called a transposase, mediates the jumping. One such transposon/transposase duo, aptly named Sleeping Beauty (SB), was found latent in the genome of salmonid fish. Its DNA sequence had mutated to the point where it no longer jumped, but rather slumbered as inactive 'junk DNA'. The extinct SB jumping functions have been resurrected², and in this issue Dupuy *et al.* (page 221)³ and Collier *et al.* (page 272)⁴ present improvements to this technology and exploit the system to identify genes involved in cancer.

Many agents, including chemicals, radiation and viruses, are routinely used to disrupt genes randomly, with the aim of identifying gene functions and the diseases associated with them. But it can be extremely difficult to find where a random mutation has been introduced, requiring huge amounts of sequencing to pinpoint each tiny change. Transposable elements are powerful reagents in this regard because their sequence is known, so when they mutate genes they provide a 'tag' that pin-

points their location in the vast sea of genomic DNA. Tagging genes by insertion is not a new idea, and geneticists working on many organisms use transposable elements. They have been little used in mice, however, because known transposons hop around the mouse genome very infrequently, yielding few new mutations.

The two groups surmounted this problem in two ways. Collier *et al.*⁴ engineered an SB transposon (T2/Onc) to have the ability to enhance or disrupt genes. They used this in a strain of mice that produce the SB transposase in all their cells and which have mutations that make them especially susceptible to cancer (Fig. 1). Dupuy *et al.*³ redesigned the transposon to be smaller (T2/Onc2), and created a mouse strain whose cells contained increased amounts of transposase, leading to higher mutation rates. A major advantage of the SB system over viral or previously used transposon systems is that, because the transposon comes from fish, it is distinct from the large number of native transposons present⁵, making it easy to find the exact site(s) in the genome where SB integrates.

The authors used their system to search for genes that cause cancer, as genes identified in mouse cancer are often also perturbed in human cancer. To understand how SB can

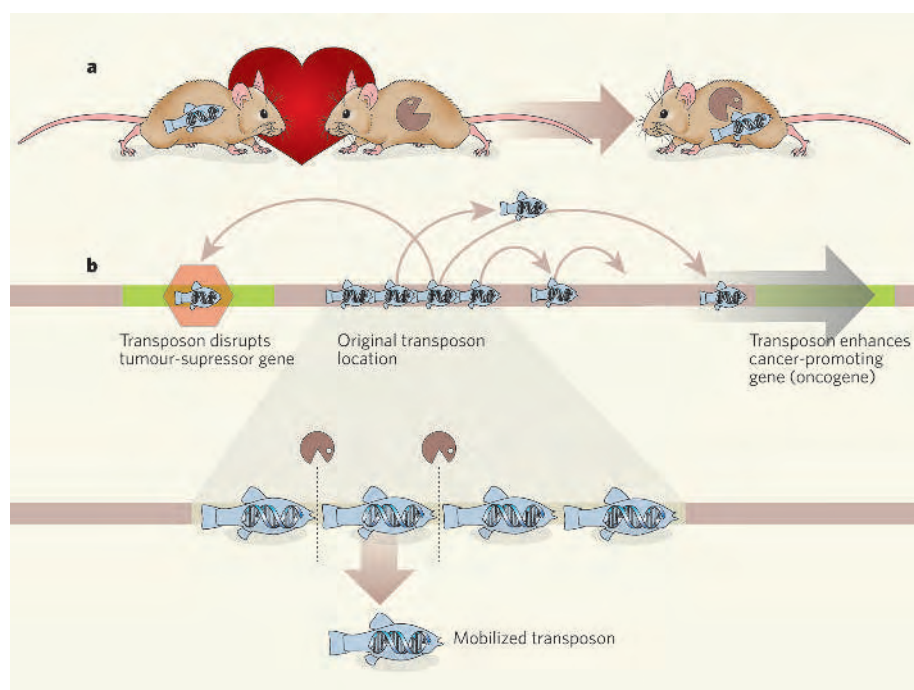


Figure 1 | DNA leaps out of fish and into cancer research. **a**, Mice whose cells contain many copies of the Sleeping Beauty transposon from fish are bred with mice whose cells make the SB transposase at high levels. Cells in the offspring contain both the transposon and transposase (pacman), allowing the transposon to hop about the genome. **b**, The transposase binds to and excises the transposon from its starting location in the genome. The excised transposon can reintegrate elsewhere in the genome, sometimes near to or within a cancer-related gene. If the transposon inserts in a gene, it will truncate the encoded protein, usually destroying its function. This will identify genes that help to protect against cancer (tumour-suppressor genes). Inserting near a gene causes an increase in gene product. This will identify cancer-promoting genes (oncogenes).

identify cancer genes, imagine the genome as a book of instructions on how cells work. SB is like a small phrase or set of directions that can hop into, and potentially alter, any instruction in the book. Sometimes, inserting the phrase will cause insignificant changes, but if SB alters instructions for key processes, such as cell proliferation or cell death, the cells can grow and divide beyond their normal potential and become cancerous. The authors designed the SB transposon to disrupt gene function in two ways. If it inserts in a gene, it will truncate the protein encoded by that gene, usually destroying its function. This will identify genes that help to protect against cancer (tumour-suppressor genes). If the transposon inserts near a gene, it causes an increase in the gene product, allowing cancer-promoting genes (oncogenes) to be identified.

By isolating the sites of SB insertion in tumours, the groups tagged genes that are known to be important in the development of cancer and those likely to be involved in the disease that had not previously been associated with it. Dupuy *et al.*³ also demonstrated networks of genes that interact to cause cancer. In addition, Collier *et al.*⁴ show, using animals that harbour cancer-predisposing mutations, that the SB system can tag genes in a solid tumour called a sarcoma. This tumour can involve various tissue types, including neural cells and connective tissue cells.

Cancer is rarely caused by the mutation of a

single gene; rather, perturbations of several genes tend to cooperate to cause the disease⁶. Genetic pathways involved in the development of leukaemia have been dissected by tagging with mouse leukaemia retroviruses^{7,8}, but gene networks in other tumour types are less well studied. The development of new treatment strategies would ideally require information on all the genes involved in common and devastating cancers such as breast, colon, prostate and lung cancer, and the SB system seems a promising way to provide this.

The technology is likely to be very powerful, because the transposase can be designed to be expressed selectively in a specific cell type or developmental stage, so that transposition will occur only in those cells or at that time. Many cancer-associated genes are disrupted in only one particular cancer, and the selective SB technique can be used to locate these. Furthermore, the ability to limit transposase expression will enable the system to be turned on to make mutations and then turned off to stabilize them. It also allows for the controlled excision of the transposons, so that mutations can be reversed.

At present, however, the ability to restrict gene expression to each type of tissue-specific stem cell is limited⁹. (Tissue-specific stem cells are the immature cells that give rise to specialized tissues, where cancer mutations are most likely to occur.) Further research into stem-cell-restricted gene expression



50 YEARS AGO

Another milestone was reached in the history of the European Organization for Nuclear Research, when on June 10 the foundation-stone was laid of the laboratories at the Meyrin headquarters of the Organization near Geneva, followed by the signature of an important Agreement between the Organization and the Swiss Government... Those who have been closely associated with the years of planning came away feeling that this unique Organization has good reason to be proud of what has been achieved so far. The high level of scientific and technical competence of the research and design teams and the spirit of enthusiasm which animates all concerned provide solid grounds for confidence in the healthy growth of the new Organization. From *Nature* 16 July 1955.

100 YEARS AGO

"The popularisation of science." *The New Knowledge* by Robert Kennedy Duncan. The author of this attempt to make the progress of recent discovery in chemistry and physics understood of the people remarks in his preface:—"The great expositors are dead, Huxley and Tyndall and all the others; and the great expositor of the future, the interpreter of knowledge to the people, has yet to be born." And (but it must be added quite modestly) he attempts to wear the cloak of prophet... To give the reader an idea of the author's style, a quotation from the first paragraphs of part ii may be made... "Here, for example, is a swarm of atoms, vibrating, scintillant, martial,— they call it a soldier,— and, anon, some thousands of miles away upon the South African veldt, that swarm dissolves,— dissolves, forsooth, because of another little swarm,— they call it lead."... Now this purports to be very fine writing, but does it gild the pill of science? I am inclined to think not. Still, tastes may differ. From *Nature* 13 July 1905.

50 & 100 YEARS AGO

will expand the application of the SB system.

The SB system of genome manipulation and mutation will be valuable in organisms other than mice, and has applications outside cancer. It can be used in human cells, or in any organism that is a model for human disease. The transposon can be engineered to deliver any DNA cargo to many locations in the genome. This has remarkable potential for discovering the genes associated with diseases that have a genetic component (heart disease, diabetes, birth defects, and many more). It might also provide therapeutic gene delivery and large-scale genome modification. With the genome sequences for many organisms nearly complete, the utility of this beautiful tag for DNA mutations is infinite. ■

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PARTICLE PHYSICS

Weighty questions

Ian Shipsey

In an unprecedented feat of computation, particle theorists made the most precise prediction yet of the mass of the ‘charm–bottom’ particle. Days later, experimentalists dramatically confirmed that prediction.

The lofty endeavour of particle physicists — to understand the birth, evolution and ultimate fate of the Universe by studying its fundamental particles — has just received a significant boost. The fiendishly difficult equations of the strong nuclear force have yielded to a 30-year effort to allow the first precise prediction of a composite particle’s mass¹, a prediction promptly confirmed by experiment². The computational technique responsible, lattice quantum chromodynamics, could also be used to estimate quark masses better, to shed light on the origin of mass, and to reveal how the Universe, originally made of matter and

antimatter in equal proportions, ended up containing just matter.

The ‘standard model’ of particle physics describes the interaction of fundamental particles, such as quarks and electrons, as the exchange of other particles that convey force. In an atom, for example, electrons bind to protons by swapping massless photons — the familiar electromagnetic force encompassed by the theory of quantum electrodynamics (QED). Similarly, inside the proton, ‘up’ and ‘down’ quarks bind by exchanging massless particles called gluons — an interaction described by the so-called strong force and the

theory of quantum chromodynamics (QCD). Without this strong force, which not only binds quarks to form protons, but also keeps protons together in the nucleus, matter would simply fall apart.

The fundamental particles possess a wide range of masses: an electron is about 360,000 times lighter than the heaviest, or ‘top’, quark. In the standard model, the origin of the mass of fundamental particles is the yet-to-be-discovered Higgs force field. The reason electrons and quarks have the exact masses that they do must, however, come from a deeper theory of nature. Physicists hope that gaining knowledge of the quark masses will guide them to that theory.

As quarks are permanently bound into composite particles (Fig. 1), it is not possible to determine their masses directly. Theorists instead solve the equations of QCD for a composite particle made up of quarks (protons and neutrons are examples) with the quark masses and strength of the strong force as unknowns. The most likely value of a quark mass is that which best reproduces the measured mass of the composite particle. As energy is related to mass through $E = mc^2$, where m is the mass of the particle and c the speed of light, this mass depends not only on the mass of the constituent quarks, but also on the bonds between them (potential energy) and their motion (kinetic energy).

The mass of a hydrogen atom is less than the mass of its constituents by the electromagnetic binding energy (–13.6 electronvolts) — an effect of 1 part in 100 million. The effect of the strong force is greater: the mass of a nitrogen nucleus, with seven protons and seven neutrons, is less than the mass of its constituents by a binding energy of –105 mega-electronvolts (MeV), 0.7% of the total mass. This is the formidable energy that is released in nuclear fission and fusion reactions.

The binding effects between quarks are fundamentally different from those in atoms and

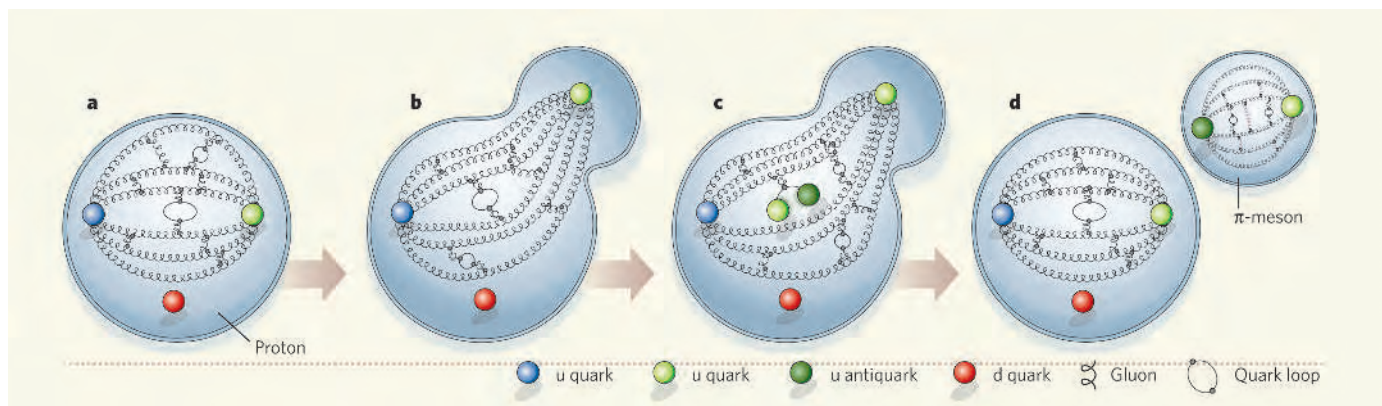


Figure 1 | Gregarious quarks. **a**, Free quarks do not exist. In a proton, for example, the three principal quarks — two ‘up’ (u) and one ‘down’ (d) — are bound with a force of more than 10 tonnes by the exchange of gluons (curly lines). Additional gluons and quark pairs are constantly emitted, only to be reabsorbed; just a fraction of this swarm of particles is shown here, and for clarity the gluons that pass between the d quark and u quark in the proton are not shown. **b**, Tug on one of the three quarks in a proton, and **c**, by the time the separation is a proton radius (about 10^{-15} m), enough work has been done to produce a quark and an antiquark. **d**, The antiquark latches on to the quark to form a quark–antiquark state called a π -meson; the new quark replaces the original in the proton. The ‘charm–bottom’ particle, the mass of which was predicted by Allison *et al.*¹, is a heavier variant of the π -meson, containing a ‘charm’ quark and a ‘bottom’ antiquark.

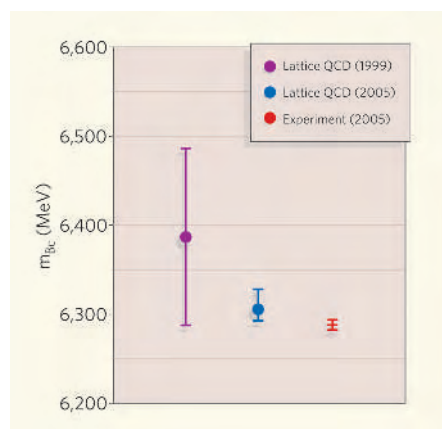


Figure 2 | Perfect harmony. A comparison of the new lattice QCD calculation of Allison *et al.*¹ (central point) for the mass of the B_c meson compared with an earlier calculation⁷ (left) and the experimental measurement of Acosta *et al.*² (right). The uncertainty in the new calculation is five times smaller than the previous one and is validated by the experimental measurement.

atomic nuclei: they are positive, and so increase the mass of the composite particle. They are also two orders of magnitude larger than the quark masses themselves. In other words, nearly all the mass of protons and neutrons — of ordinary matter in the Universe, including stars, planets and humans — is due to the strong force's binding energy.

A precise calculation of the electromagnetic binding energy for hydrogen must include the possibilities that photons can be emitted and reabsorbed by the electron, and that pairs of electrons and positrons (the antimatter counterpart of the electron) can pop briefly into existence. Because the electromagnetic force is weak, these quantum corrections are small; a calculation involves just adding up a sufficient number of them. Consequently, the theory of QED has been verified to the tenth decimal place.

QCD is more complicated: not only do pairs of quarks and antiquarks make fleeting appearances and quarks constantly exchange gluons, but those gluons can constantly exchange other gluons as well. The quantum corrections are so large that adding them all up is not feasible. So the strong-force properties of composite particles are instead calculated using powerful computers to keep track of the most probable arrangements of the quarks and gluons inside them. No computer in existence can follow every quark and gluon, so the problem is simplified by imagining space and time not as a continuum, but as a lattice — a four-dimensional grid of discrete points in space-time. Quarks reside at these points and gluons on the links between them, reducing an infinite number of variables to a finite (though very large) number — an approach known as lattice QCD.

In the 1980s, lattice QCD was used to explain why quarks are bound inside protons. But the first realistic calculation of particle properties, rather than prediction of qualita-

tive features, came only in 2003. Then, a significant breakthrough³ in the lattice technique — and teraflop-scale computers running for two years — allowed the inclusion of all pairs of light quarks (up, down and a third, slightly heavier variety, 'strange') and antiquarks that fluctuate into brief existence inside a composite particle. These quark-antiquark pairs had usually been left out of the calculations because their simulation — much more difficult than that of gluons — had demanded prohibitively large amounts of computer time.

After first obtaining the quark masses by 'tuning' the lattice to reproduce the masses of some well-known composite particles, ten further strong-force properties of composite particles were calculated³. The deviation of the calculated values from the accepted experimental values was never more than a few per cent. But the first prediction of an unknown quantity was still wanting; it is this that a team of lattice QCD experts from Glasgow University in Scotland and Ohio State University and Fermilab in the United States, writing in *Physical Review Letters*¹, has now supplied.

The composite particle B_c or 'charmed B-meson', a bound state of a charm quark and a bottom antiquark, is known as the 'last meson' because it was the final such quark-antiquark pairing that physicists expected to find. The B_c was discovered in 1998, but its mass could not be determined accurately. Here was thus a rare opportunity for the lattice theorists to predict the mass before better measurements were made. Only days after their prediction¹ of $6,304 \pm 22$ MeV appeared on a preprint server, the CDF experiment², picking through the pieces of trillions of particle collisions at Fermilab's Tevatron accelerator in Illinois, isolated 19 examples of the last meson with a mass of $6,287 \pm 4.9$ MeV. The agreement between theory and experiment (Fig. 2) is a powerful validation of the lattice technique, especially for bottom and charm quarks.

An even stiffer test is imminent. Quarks also experience radioactive decays through the so-called weak force, the third fundamental force of the standard model. The pattern of these decay rates would be, especially for bottom quarks, sensitive to phenomena beyond the standard model⁴. The binding effect of the strong force between quarks modifies the decay rates, and so correction factors are needed to allow a full interpretation of new data.

These correction factors are even more difficult to predict than the composite particle masses. But their first calculation⁵ for mesons containing charm quarks has just been performed. Almost simultaneously, the CLEO experiment at Cornell University in New York has announced the result⁶ of the first measurement. The agreement is again good, although the accuracy of both the prediction and the experimental value is only around 10% (this should improve to under 5% within a year). If the agreement persists, similar calculations can be confidently applied to correct measurements of the disintegration rates of bottom quark mesons, whose correction factors are not measurable. If the corrected rates do not conform to standard-model predictions, they could provide hints about the deeper theory that gives quarks their mass — and, ultimately, why only matter exists in the Universe. ■

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PHYSICAL CHEMISTRY

Trapped gas

G rard F rey

Acetylene is a gas with many industrial applications. A highly efficient method to separate it from its close relation, carbon dioxide, is a promising route for purifying and storing 'strategic' gases in general.

Technologies for the separation and storage of gases are essential for producing clean fuels based on methane and hydrogen, for example, or for reducing air and water pollution from poisonous gases such as the oxides of carbon, nitrogen and sulphur¹. Kitagawa and colleagues (Matsuda *et al.*), writing on page 238 of this issue², provide a full characterization of a porous hybrid solid that retains the gas

acetylene — itself a key material in the chemical and electronics industries — at room temperature and pressure, but adsorbs carbon dioxide only at high pressures and low temperatures. The possible extension of their methods to other strategically important gases makes their paper an outstanding contribution to this field of research.

Work on gas adsorption — the capacity of a

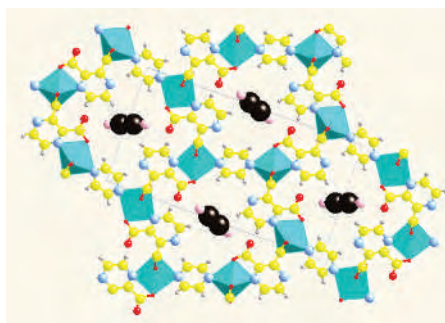


Figure 1 | Acetylene caught. A view of the porous hybrid organic-inorganic solid $\text{Cu}_2(\text{pzdc})_2(\text{pyz})$ used by Kitagawa *et al.*² to trap acetylene (C_2H_2). The trapped acetylene molecules (black, carbon; pink, hydrogen) are shown within the hybrid skeleton (yellow, carbon; pale blue, nitrogen; red, oxygen; grey, hydrogen; bright blue polyhedra, copper).

solid (the adsorbent) to bind foreign gas molecules (the adsorbate) to its surface — has already provided useful results for methane and hydrogen^{3,4}. Among the materials currently used as generic adsorbents are activated carbons, zeolites and nanotubes. The fabrication of high-capacity adsorbents tailored to particular adsorbates, however, requires a better knowledge of adsorbate-adsorbent interactions at the atomic level.

In this context, the search for porous hybrid inorganic-organic solids is expanding rapidly^{5,6}. Such solids, in which organic and inorganic molecular groups react to form a porous skeleton, have been identified as the best candidates for efficient interaction with gases because their pore dimensions and surface structure⁶ can be tuned by changing the organic linker molecule used. This implies potential applications not just in gas separation and storage, but also in catalysis. Adsorption occurs on the internal surface of the pores, usually through the onset of van der Waals forces between adsorbate and adsorbent (a process known as physisorption).

The amount of a gaseous species that can be adsorbed by a porous solid increases as the pressure of the gas rises, and decreases as its temperature rises. The rate of adsorption is dependent on the chemical affinity of the gas

molecule for the solid wall. The technique of gas separation depends on the differing affinities of the various gas molecules for the wall: for a mixture of molecules, the larger the difference in their time of retention on the surface, the better the separation that can be achieved. The separation is very selective if, in a two-gas mixture, one gas species has an infinite time of residence on the surface — it becomes trapped — and the other gas species escapes the structure as the pressure of the remaining gas mixture decreases and the temperature rises.

Such a situation occurs in Kitagawa and colleagues' study² to separate a mixture of acetylene and carbon dioxide. The adsorbent solid they describe (Fig. 1) has the formula $\text{Cu}_2(\text{pzdc})_2(\text{pyz})$, where pzdc and pyz are two organic groups, pyrazine-2,3-dicarboxylate and pyrazine, respectively. The solid's three-dimensional structure captures acetylene (C_2H_2) at 300 kelvin — just above room temperature — whereas carbon dioxide, which has the same external dimensions as acetylene and very similar bulk properties, and which is a common impurity in industrial processes, exhibits a classical, reversible adsorption-desorption behaviour. At a pressure as low as 1.1 kilopascals (about a hundredth of atmospheric pressure), around 26 times more C_2H_2 is adsorbed than CO_2 . From an accurate structure determination at various temperatures between 270 and 340 kelvin, Kitagawa *et al.* localize the C_2H_2 molecules in the solid, and establish their interaction with sites in the wall, from both structural and first-principles calculations.

The first striking feature of Kitagawa and colleagues' work² is its quality — it combines creativity, accurate characterization and energy calculations to demonstrate the incarceration behaviour of acetylene, and it also explains that behaviour. The authors use a technique known as MEM analysis to establish the electron density through the C_2H_2 molecule. They show that, unlike processes involving other adsorbed species that imply the presence of weak van der Waals forces, the C_2H_2 -wall interaction proceeds through the interplay of acidic protons in the adsorbate

and alkaline oxygen in the absorbent (Fig. 2). This highlights a point generally neglected until now: that the nature of the bonds in the adsorbate can also influence adsorption.

The second striking feature of the findings is the stoichiometric 1:1 ratio between the number of adsorbed C_2H_2 molecules and the number of available pores, which is reached very quickly at the lowest pressures. In contrast, a slow, smooth increase in the amount of CO_2 adsorbed occurs as the pressure is increased. The case of acetylene is reminiscent of the situation when water is the adsorbate, where there is a whole-number molecular ratio between guest and host. Could this be a required condition for incarceration?

More generally, Kitagawa and colleagues' work² poses new questions and opens novel perspectives. For example, what is the physical meaning of adsorption at the atomic level? The macroscopic 'adsorption isotherms' (graphs of adsorption capacity as a function of pressure at a constant temperature), and the related surface areas that they measure, are based on models of monolayer coverage of the surface by molecules — a result that, combined with others⁷, implies that adsorption corresponds to the fixation of molecules only on some sites of the wall. Further studies like those of Kitagawa *et al.*, as well as computer simulations⁸, are required to identify the adsorption sites in porous hybrid organic-inorganic solids. This will probably lead to a more sophisticated definition of surfaces that takes into account the discontinuous nature of adsorption at the atomic level.

Kitagawa and colleagues stress that in their study the size of the adsorbate and that of the absorbent's pores are similar. Does this mean that there is an optimal size of pore for fixing a given molecule? Or are larger pores^{6,9} generally beneficial? At a time when attempts are being made to predict the structure of solid hybrids¹⁰, the next step, suggested by this study, will be to predict the structure of the active adsorption sites themselves. Clearly, finding the next set of answers to questions of gas storage and separation is a long-term challenge. But the potential of these answers to help in developing sustainable technologies for the benefit of humankind and the environment make this a challenge worth tackling. ■

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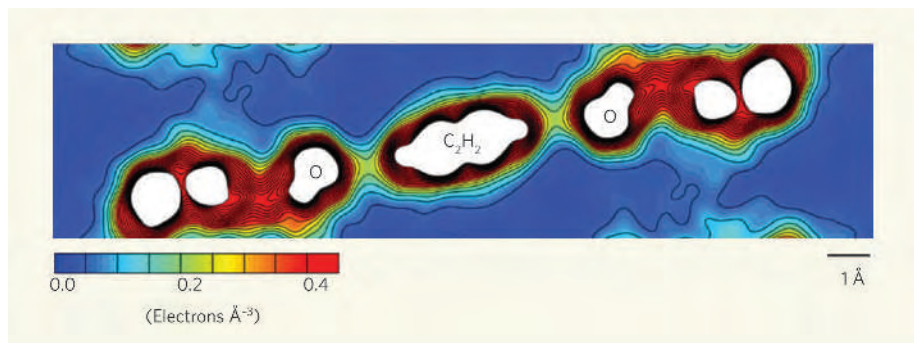


Figure 2 | Acid-base interaction. A projection of the electron-density distribution on the two-dimensional section along the molecular axis of C_2H_2 . The electron density decreases from white to blue (see scale) through red and yellow. The continuum (yellow) of electron density between the C_2H_2 molecule and the oxygens of the framework indicates a noticeable acid-base interaction.

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ENTOMOLOGY

Incompatible mosquitoes

Ary A. Hoffmann

Infection of mosquitoes by a particular bacterium has a fiendishly complicated influence on the success or failure of mosquito breeding. A window now opens on the molecular basis of this 'cytoplasmic incompatibility'.

Cytoplasmic incompatibility (CI) is a well-known phenomenon in many insects and mites¹. It occurs when an agent inherited in the cell cytoplasm causes matings between strains of the same species to yield few or no offspring, and it is usually associated with infections of a bacterium known as *Wolbachia*. The bacterium lives inside the reproductive cells of its host, and was first associated with CI in a mosquito, *Culex pipiens*, when it was discovered that treating different strains of this species with an antibiotic restored compatibility between them. However, there are complex patterns of cross-compatibility between strains of *C. pipiens* from different populations², and these have never been explained.

On page 257 of this issue, Sinkins *et al.*³ describe how they have used information from *Wolbachia* genome-sequencing projects to link this complexity to genes encoding specific protein motifs — members of the ankyrin family. This research opens up opportunities for understanding both the biological basis of CI and the potential for enlisting *Wolbachia* for mosquito control.

In its simplest form, CI is unidirectional — that is, with two strains of individuals involved in incompatibility, characterized by being either infected or uninfected by a single type of *Wolbachia*. Infected females are compatible with uninfected and infected males, but when uninfected females mate with infected males the embryo dies (Fig. 1). Because infected females mate successfully with any male and transmit the infection to all of their offspring, the *Wolbachia* infection should spread and occur at near 100% in populations. These dynamics apply to a *Wolbachia* infection in *C. pipiens* from California⁴.

To generate other forms of incompatibility among strains, different types of *Wolbachia* are needed. CI can be bidirectional when strains carry different *Wolbachia* infections. There is incompatibility when males with one type of infection mate with females carrying a different infection, which means that these strains cannot reproduce successfully (Fig. 1). When the same individual carries two different *Wolbachia* infections, complex patterns of incompatibility can occur. Males carrying both infections can show unidirectional CI not only with uninfected females, but also with females carrying only one of the infections. Moreover, strains with only one type of *Wolbachia* can show bidirectional CI. Double infections that act in this manner are

		FEMALE			
		Uninfected	A	B	AB
MALE	Uninfected	✓	✓	✓	✓
	A	✗	✓	✗	✓
	B	✗	✗	✓	✓
	AB	✗	✗	✗	✓

Figure 1 | Types of cytoplasmic incompatibility (CI). A and B are *Wolbachia* infections that occur singly (A, B) or together (AB) in cells. The blue area represents unidirectional CI, the green area bidirectional CI; the A × A mating is part of both unidirectional CI and bidirectional CI. Crosses indicate incompatibility, and ticks compatibility. A complex pattern of incompatibility results when strains with different *Wolbachia* status are intercrossed.

found in mosquitoes such as *Aedes albopictus*⁵.

Although complex patterns of CI occur in crosses between strains of *C. pipiens*, attempts to isolate different types of *Wolbachia* from these strains have previously failed, even when using variation in genes known to distinguish *Wolbachia* infections in other insects⁶. Sinkins *et al.*³ have now shown that *C. pipiens* strains that are bidirectionally incompatible differ in two ankyrin-encoding genes. Ankyrins are short, repeated amino-acid motifs thought to mediate interactions among proteins, as well as between proteins and the cytoskeleton, the cellular scaffolding. Genes with ankyrin sequences are particularly common in the *Wolbachia* genome relative to those of other bacteria⁷, making them candidates for the expression of different patterns of CI, especially as they may control the way *Wolbachia* interacts with the cell cycle of its host.

Sinkins *et al.* found substantial genetic divergence in the two ankyrin-encoding genes of the two *C. pipiens* strains they examined; almost 10% of the nucleotides had diverged for one gene and around 3% for the other. The same genes also differed in another strain that exhibits a different incompatibility pattern. So these genes may be responsible for the varied expression of incompatibility, although the mechanism remains unknown.

Further players in events need introducing here. These are bacteriophages, phages for

short, which are viruses that infect bacteria and can insert their genomes into that of their host. They enter the story because it turned out that both of the ankyrin-encoding genes are located in regions of the *Wolbachia* genome where phage chromosomes are inserted. Phages can excise from these regions and carry genes located within them to other places in the same genome or different genomes.

By using filters to separate the small phage particles from the larger *Wolbachia*, Sinkins *et al.*³ found that the two ankyrin-encoding genes could be carried by phage particles independently of the *Wolbachia* genome. This is the first time that putative genes associated with CI expression have been located on phage particles, although it has previously been suspected⁸ that phages may be involved in CI. Phage activity, along with the movement of transposable genetic elements, may help to explain the marked changes in the arrangement of ankyrin genes among *Wolbachia* genomes from related hosts⁹. Variation in one such element, known as Tr1, has also been associated with different incompatibility strains of *C. pipiens*¹⁰.

Wolbachia have long been promoted as potential vehicles for transferring deleterious genes into mosquitoes, and other insect populations, for pest control. Among the obstacles to this is the absence of a system for genetically transforming *Wolbachia* and a way of generating new CI types capable of spreading transgenes. If CI genes are located within phage chromosomes, this should help in developing *Wolbachia* infections that can spread deleterious genes.

But there remains a danger that changes in the host's nuclear genome will decrease the efficacy of CI as a mechanism to drive transgenes into mosquito populations. Indeed, Sinkins *et al.*³ used backcrosses of two strains of a mosquito species related to *C. pipiens* to show that nuclear genes modified the expression of incompatibility, highlighting the fact that these types of evolutionary changes are possible. So although we are now closer to understanding CI and using *Wolbachia* to introduce transgenes, host genome evolution may yet be a barrier to the aim of using *Wolbachia* for suppressing pest populations. ■

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BRIEF COMMUNICATIONS

H5N1 virus outbreak in migratory waterfowl

A worrying development could help to spread this dangerous virus beyond its stronghold in southeast Asia.

The highly pathogenic H5N1 influenza virus has become endemic in poultry in southeast Asia since 2003 and constitutes a major pandemic threat to humans¹. Here we describe cases of disease caused by H5N1 and transmission of the virus among migratory geese populations in western China. This outbreak may help to spread the virus over and beyond the Himalayas and has important implications for developing control strategies.

H5N1 virus has occasionally been isolated from dead wild birds, usually within the flight range of infected poultry farms^{2,3}. In the absence of evidence that the virus is transmitted within wild bird populations or that migratory birds can carry the virus, it was possible that these birds were dead-end hosts of virus acquired from poultry. On 30 April 2005, however, an outbreak was detected in bar-headed geese (*Anser indicus*) at Qinghai Lake in western China (see supplementary information), which is a protected nature reserve with no poultry farms in the vicinity.

Initially, sick bar-headed geese were recorded on a single islet that contained about

3,000 bar-headed geese as well as some brown-headed gulls (*Larus brunnicephalus*), great black-headed gulls (*Larus ichthyaetus*) and great cormorants (*Phalacrocorax carbo*). Clinical findings included paralysis, unusual head tilt, staggering and neck thrill — all are known features of H5N1 disease in waterfowl. By 4 May, bird mortality was more than 100 a day; by 20 May, the outbreak had spread to other islets, with some 1,500 birds dead.

Overall, 90% of the dead birds were bar-headed geese, with the remainder being brown-headed gulls and great black-headed gulls. We isolated 28 H5N1 viruses from 92 cloacal, tracheal and faecal swabs from all three species, and a further 5 viruses from tissue samples from bar-headed geese. (For details of methods, see supplementary information.)

Sequence comparison revealed that the H5N1 viruses were almost identical across all gene segments. The haemagglutinin gene retains the motif of basic amino acids (QGER-RRKKR) in the connecting peptide that characterizes highly pathogenic avian flu. All Qinghai isolates had a Lys 627 mutation in the PB2 gene,

which has been associated with increased virulence in mice⁴. Phylogenetic analysis of these isolates and eight other H5N1 viruses, isolated from poultry markets in Fujian, Guangdong, Hunan and Yunnan provinces during 2005, indicated that the haemagglutinin (Fig. 1a), neuraminidase and nucleoprotein (data not shown) genes of the Qinghai viruses were closely related to the H5N1 virus A/Chicken/Shantou/4231/2003 (genotype V).

However, the other five internal genes, represented by the matrix-protein gene, were closely related to H5N1 viruses isolated from domestic poultry in southern China during 2005, represented by the virus A/Chicken/Shantou/810/2005 (genotype Z) (Fig. 1b). These viruses are therefore characterized as H5N1 genotype Z, but are clearly distinguishable from those that have caused human infection in Thailand and Vietnam (Fig. 1a, b)⁵. This indicates that the virus causing the outbreak at Qinghai Lake was a single introduction, most probably from poultry in southern China.

Qinghai Lake is an important aggregation and breeding site for bar-headed geese that are distributed over central Asia⁶. From September, they migrate southwards to Myanmar and over the Himalayas to India, returning to Qinghai around April⁶. Our findings indicate that H5N1 viruses are now being transmitted between migratory birds at the lake. Although the outbreak could burn itself out, the large migratory bird population at Qinghai Lake makes this unlikely. The viruses might also move to other migratory species that could act as carriers, remaining highly pathogenic for domestic chickens and possibly humans.

Like its precursor, A/Goose/Guangdong/1/96, the current H5N1 virus could become established in bar-headed geese. There is a danger that it might be carried along the birds' winter migration routes to densely populated areas in the south Asian subcontinent, a region that seems free of this virus, and spread along migratory flyways linked to Europe. This would vastly expand the geographical distribution of H5N1. Increased surveillance of poultry is called for because previous experience has shown that control measures become almost impossible once the virus is entrenched in poultry populations.

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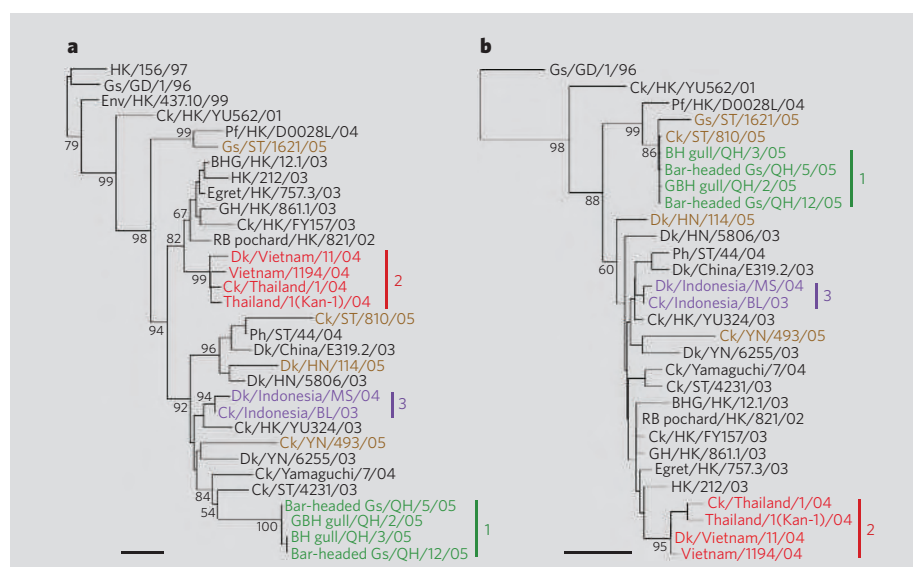


Figure 1 | H5N1 flu strains in wild birds in western China. **a**, **b**, Phylograms showing the genetic relationship between representative strains for **a**, the haemagglutinin gene (nucleotide positions 25–1,025; scale bar, 0.01 nucleotide changes per site), and **b**, the matrix-protein gene (nucleotide positions 91–956; scale bar, 0.01 nucleotide changes per site). Numbers at branches are bootstrap values from 1,000 replicates. Sources of isolated viruses: clade 1, Qinghai Lake; clade 2, Thailand and Vietnam; clade 3, Indonesia; viruses isolated from southern China in 2005 are shown in brown. BH gull, brown-headed gull; BHG, black-headed gull; Ck, chicken; Dk, duck; Env, environment; GBH gull, great black-headed gull; GD, Guangdong; GH, grey heron; Gs, goose; HK, Hong Kong; HN, Hunan; Pf, peregrine falcon; Ph, pheasant; RB pochard, rosy-billed pochard; ST, Shantou; YN, Yunnan. Sequences have been deposited in GenBank under accession numbers DQ095612–DQ095771.

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Supplementary information accompanies this communication on Nature's website.

Competing financial interests: declared none.

doi:10.1038/nature03974

Published online 6 July 2005.

CONSERVATION

Top predators and biodiversity

The charisma of top vertebrate predators is often used by conservationists as a lever for financial support^{1,2}, to raise environmental awareness^{2,3} and in planning protected areas^{4–6} — a strategy that has been criticized^{3,5,7}. Here we use information collected from five raptor species that differ widely in their diet and habitat associations to show that sites occupied by these predators are consistently associated with high biodiversity. The biodiversity at these sites is more extensive than it is at sites selected at random, or at sites occupied by species from lower down the trophic pyramid (insectivorous or herbivorous species, for example). Our results indicate that

conservation focusing on top predators can be ecologically justified because it delivers broader biodiversity benefits.

We surveyed five species of diurnal and nocturnal raptors in the Italian Alps during the breeding season: the goshawk (*Accipiter gentilis*), pygmy owl (*Glaucidium passerinum*), Tengmalm's owl (*Aegolius funereus*), tawny owl (*Strix aluco*) and scops owl (*Otus scops*) (for site locations, see supplementary information). The diversity of birds, trees and butterflies was used as a surrogate measure of biodiversity. For each raptor species, we compared the biodiversity recorded at 25 sites occupied for breeding with that at three other types of site: 25 sites randomly chosen in comparable habitat (spatial-control sites); 25 breeding sites of a randomly selected species from a lower trophic level (taxonomic-control sites A); and 25 breeding sites of a species from a lower trophic level that has specialized ecological requirements (taxonomic-control sites B). (For methods, see supplementary information.)

Compared with the three types of control site, locations occupied by top predators had greater numbers and more diversity of avian species, vulnerable avian species, butterfly species and tree species (for all raptor species and for all comparisons, $t \geq 3.14$, $P < 0.05$, except for one comparison, for which $t = 2.96$, $P < 0.07$; Fig. 1a–c). By contrast, none of the biodiversity estimates collected at sites occupied by species from lower trophic levels was higher than those collected at their associated

spatial-control sites (for all species, $t \leq 2.45$, $P \geq 0.16$; Fig. 1a–c).

We also did a simulation exercise (gap analysis^{7,8}; see supplementary information) that selects a network of protected areas by maximizing the overall level of biodiversity representation while minimizing the number of protected areas included in the network. This showed that the number of sites required to include all avian species, or all vulnerable avian species, in a hypothetical protected system was lower when using sites occupied by top predators than when using any type of control site ($t \geq 2.89$, $P \leq 0.05$). The difference between sites occupied by species from lower trophic levels and their associated spatial-control sites was not significant ($t \leq 1.64$, $P \geq 0.14$).

Furthermore, the efficiency of each protected-area system (expressed as the percentage of maximum attainable biodiversity) was higher for top-predator sites than for any type of control site ($t \geq 3.97$, $P < 0.01$; Fig. 1d), whereas it did not differ between sites that were occupied by lower-trophic-level species and their associated spatial-control sites ($t \leq 1.36$, $P \geq 0.21$; Fig. 1d). On average, networks planned using lower-trophic-level species represented 72% of the maximum species richness and 76% of the maximum richness of vulnerable species in each sample, compared with 94% and 93% for networks based on the presence of top predators.

Our results are evidence of a tight association, at least in some biological systems, between apex predators and high biodiversity, which may justify the strategic economic exploitation of top-predator species on ecological grounds. More information from other systems will be needed before the powerful top predators can be dismissed from the conservation arena as unscientific tools.

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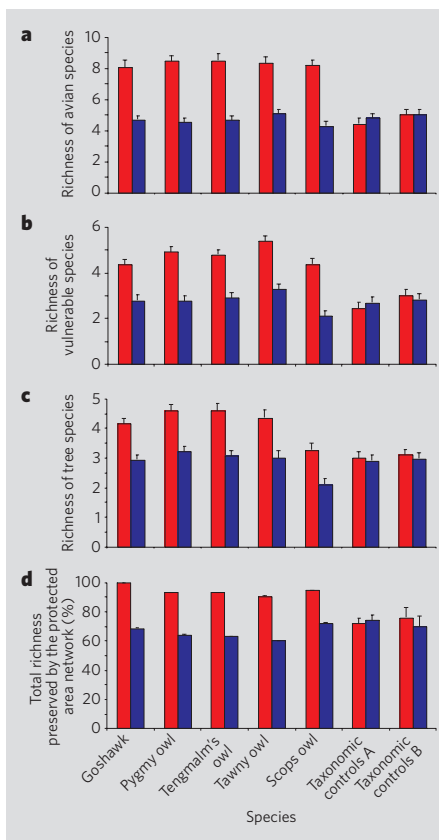


Figure 1 | Biodiversity estimates are higher at sites occupied by five top predators than at randomly selected sites or at sites occupied by species from lower trophic levels (taxonomic controls). Red bars, breeding sites; blue bars, randomly selected spatial-control sites. Values represent averages ± 1 s.e. **a**, Numbers of all avian species. **b**, Numbers of avian species classified as vulnerable. **c**, Numbers of tree species. **d**, Percentage of maximum attainable avian-species richness in a hypothetical system of protected areas, as estimated by gap analysis^{7,8}.

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Supplementary information accompanies this communication on Nature's website.

Competing financial interests: declared none.

doi:10.1038/436192a

REVIEWS

Common mechanisms of nerve and blood vessel wiring

Peter Carmeliet¹ & Marc Tessier-Lavigne²

Blood vessels and nerve fibres course throughout the body in an orderly pattern, often alongside one another. Although superficially distinct, the mechanisms involved in wiring neural and vascular networks seem to share some deep similarities. The discovery of key axon guidance molecules over the past decade has shown that axons are guided to their targets by finely tuned codes of attractive and repulsive cues, and recent studies reveal that these cues also help blood vessels to navigate to their targets. Parallels have also emerged between the actions of growth factors that direct angiogenic sprouting and those that regulate axon terminal arborization.

During evolution, organisms have come to perform more specialized tasks, requiring an increased degree of information processing by neurons and supply of nutrients by blood vessels. Wiring of neuronal axons and blood vessels into functional circuits is therefore of utmost importance. The complexity of this task is underscored by the high degree of orderly patterning of the neural and vascular networks. The choreographed morphogenesis of both networks suggests that they are directed by genetically programmed mechanisms. Five centuries ago, Andreas Vesalius illustrated the parallels in the stereotyped branching patterns of vessels and nerves (Fig. 1a, b). Today, evidence is emerging that blood vessels, which arose later in evolution than nerves, co-opted several of the organizational principles and molecular mechanisms that evolved to wire up the nervous system. In this review, we highlight these common morphogenetic signals and mechanisms, and illustrate how intricately the navigational mechanisms for both systems are intertwined.

Lining up nerves and vessels

Neural and vascular networks appear, at least superficially, to develop differently. Neurons send out a cable-like axon that migrates over often considerable distances to reach its targets. The task of leading the axon is performed by the growth cone: a highly motile, sensory structure at the axon tip^{1–3}, discovered over a century ago by Ramon y

Cajal (Fig. 2a, b). Through dynamic cycles of extension and retraction of filopodial extensions, the growth cone continually explores and reassesses its spatial environment and accurately selects a correct trajectory among the maze of possible routes. Upon reaching its target, the axon often arborizes to allow innervation of multiple target cells. In addition to this terminal arborisation, an axon may branch ‘interstitially’ before reaching its target to make connections with different target regions. Many branches are later pruned to refine the initial pattern of projections^{1,4}.

In contrast to axons, the vasculature arises through a hierarchy of mainly local movements of endothelial cells that appear superficially to have more in common with the branching morphogenesis of other tissues than axonal patterning. During initial vasculogenesis, a primitive labyrinth of similarly sized vessels forms from an assemblage of locally proliferating endothelial cells⁵ (Fig. 2c). Then, during angiogenesis, vessels sprout off more side branches (through delamination and migration of endothelial cells, rather than formation of a cellular extension, as in the case of axons) to colonize the avascular areas in the embryo. During subsequent vascular remodelling, many of the initially small vessels coalesce or expand, or instead are pruned, resulting in the emergence of the mature vascular network of larger vessels ramifying into smaller branches⁵. In the words of Aristotle: “blood vessels are like watercourses in gardens: they start from one spring, and branch off into numerous channels, and then into still

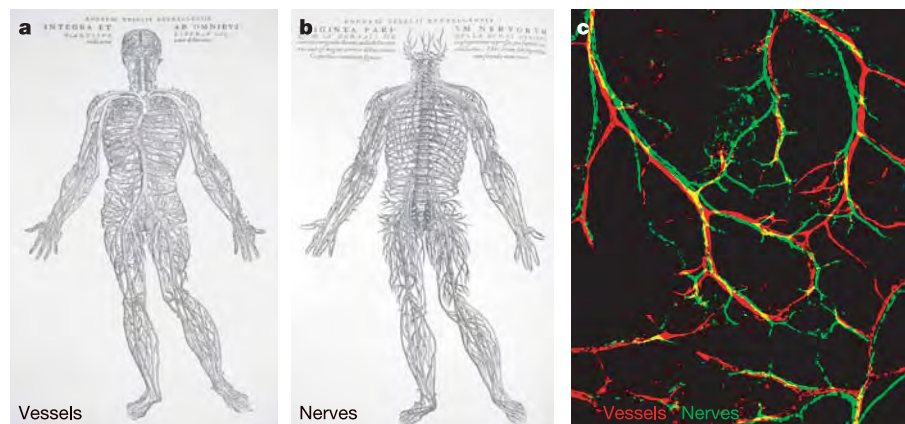


Figure 1 | Parallels in vessel and nerve patterning. a, b, Drawing by the Belgian anatomist Andreas Vesalius¹⁰¹, highlighting the similarities in the arborization of the vascular and nervous networks. c, Vessels (red) and nerves (green) track together towards their targets (reproduced with permission from ref. 9).

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more, so as to carry a supply to every part of the garden"¹⁰⁴.

The distances over which endothelial cells migrate might not be as great as those travelled by growth cones, but they nonetheless require extensive motility. A century after the discovery of the growth cone, it was recognized that specialized endothelial 'tip' cells are present at the forefront of navigating blood vessels and share many similarities with axonal growth cones⁶ (Fig. 2c, d). They extend and retract numerous filopodia in saltatory fashion to explore their environment, suggesting that they direct the extension of vessel sprouts. The key function of the tip cells appears to be to 'pave the path' for the subjacent 'stalk' endothelial cells (Fig. 2e). Tip cells proliferate minimally, whereas stalk cells proliferate extensively while migrating in the wake of the tip cell, thus permitting extension of the nascent vessel.

How do the patterns of nerves and vessels line up? Axons and vessels often take advantage of one another to follow the same path. In some cases, vessels produce signals (such as artemin and neurotrophin 3) that attract axons to track alongside the pioneer vessel^{7,8}. Conversely, nerves may also produce signals such as vascular endothelial growth factor (VEGF or VEGF-A) to guide blood vessels (Fig. 1c)⁹. The ability of axons to guide vessels, and vice versa, extends the well known facts that axons can guide other axons (early 'pioneer' axons can, in some cases, be used by later 'follower' axons to reach their targets)¹ and that different types of vessel often track together (for instance, blood vessels release VEGF-C to attract lymph vessels)¹⁰. Mutual guidance, an elegant explanation for the alignment of nerves and vessels, might not seem so surprising. What was less expected, however, was the discovery, starting in the late 1990s with neuropilins and ephrins^{11–13}, that the same cues and receptors that control axon guidance also function to pattern blood vessels. This provides a distinct mechanism to explain how axons and vessels align—by responding to common cues. At the same time, recent insights into angiogenic sprouting have also revealed marked parallels with the mechanisms regulating axonal terminal arborization. Thus, whereas the overall logic of wiring of the nervous and vascular systems might be different, steps in these processes appear mechanistically related. In this review, we discuss these parallels, starting with sprouting and arborization phenomena.

Axon terminal arborization parallels vessel sprouting

Angiogenic and axon terminal sprouting both serve the same function: to provide coverage of a target tissue. In each case, target cells direct sprouting through regulation of growth factor release. For vessels, a hypoxic tissue secretes VEGF, thus beckoning its vascularization; VEGF expression is downregulated when target

cells receive appropriate oxygen supply⁵. For axon terminals, target cells devoid of synaptic input secrete growth factors such as nerve growth factor (NGF) that similarly beckon their innervation and are downregulated when appropriate electrical stimulation of the target is successful⁴. Thus, unlike the more stereotyped patterning of larger vessels and nerves (see below), formation of capillary networks and axon terminal arborizations is non-stereotyped, but governed by the metabolic or electrical needs of the target tissue. Furthermore, in both systems sprouting requires not merely the presence of growth factors, but also of appropriate gradients of these factors—we review this first for VEGF-mediated stimulation of angiogenesis.

VEGF is a key regulator of angiogenesis in health and disease^{14,15}. It stimulates division and migration of endothelial cells, and is critical for vascular development^{16,17}. In addition, VEGF is critical for the guidance of endothelial cells to their targets. Indeed, VEGF exists in different isoforms, with distinct affinities for the extracellular matrix. Thus, VEGF₁₂₁ is diffusible, VEGF₁₈₉ binds to the matrix, whereas VEGF₁₆₅ has an intermediate profile (in mice, all VEGF isoforms are shorter by one residue). By virtue of their distinct affinities, the isoforms produce a gradient, with VEGF₁₂₀ acting over a long range and VEGF₁₈₈ over a short range^{6,18} (Fig. 3a). In the mouse retina, a gradient of matrix-bound VEGF produced by astrocytes guides endothelial tip cells, as alteration of the gradient by either loss- or gain-of-function manipulations leads to loss or increased branching of vessels, respectively⁶.

Further evidence for a role of VEGF gradients in tip cell guidance was deduced from the analysis of three mouse lines (the VEGF₁₂₀, VEGF₁₆₄ and VEGF₁₈₈ lines), each of which is engineered to express a single VEGF isoform. VEGF₁₆₄ mice are normal, but VEGF₁₂₀ and VEGF₁₈₈ mice exhibit serious vascular remodelling defects^{19,20}. Vessels in VEGF₁₂₀ mutants are enlarged, stunted and exhibit reduced branching. Their tip cell filopodia extend chaotically in all directions, which is thought to cause lumen enlargement at the expense of directed branch formation and elongation (Fig. 3a). These defects presumably result from replacement of the normal VEGF gradient by a non-directional deposition of the highly diffusible VEGF₁₂₀. In VEGF₁₈₈ mice, a shortage of diffusible VEGF causes the opposite phenotype: that is, supernumerary branches at the expense of luminal enlargement (Fig. 3a).

Terminal arborization of axons is similarly directed by gradients of target-derived factors. In the case of axons, a variety of different growth factors are involved, reflecting the need for specificity in the interactions of particular target cells with distinct axons. We focus here on the role of NGF in patterning sympathetic and some sensory

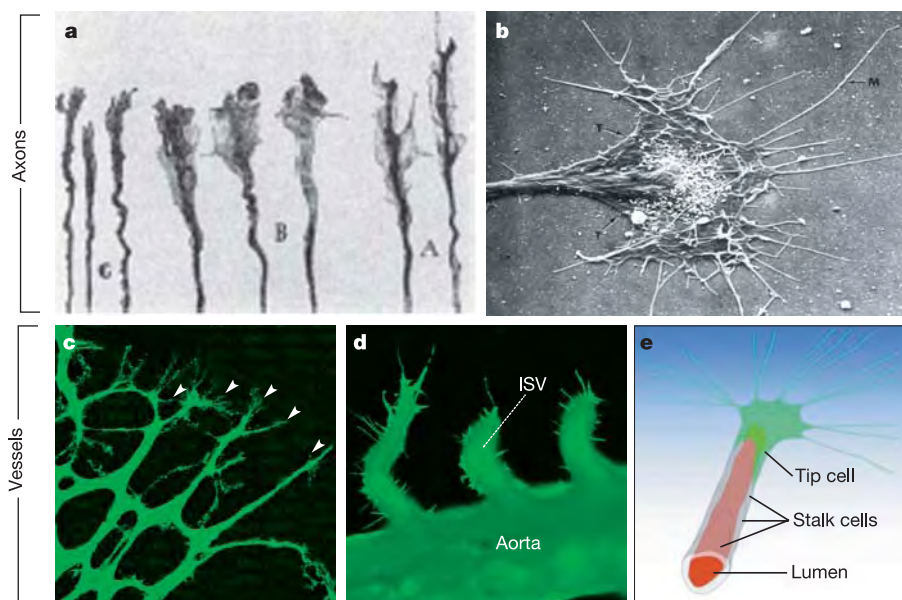


Figure 2 | Stereotyped axon and vessel navigation. **a**, Photographs of axons terminating in a growth cone, containing numerous filopodia¹⁰². **b**, Scanning electron micrograph of an axonal growth cone with characteristic filopodial extensions (from ref. 103). **c**, Primitive vascular labyrinth of similarly sized vessels in the retina of a neonatal mouse. Note how the tip endothelial cells at the leading front of the growing blood vessels extend multiple filopodia (arrows) into the avascular retina. **d**, Stereotyped pattern of intersomitic vessels (ISVs), branching off from the dorsal aorta in a 22-h-old zebrafish embryo. Note the numerous filopodial extensions at the leading endothelial tip cells. **e**, Schematic representation of endothelial tip and stalk cell (reproduced with permission from ref. 6). (Labels C, B, A in **a** and T and M in **b** indicate details not discussed here.)

connections. NGF regulates target innervation by sensory and sympathetic axons in multiple ways²¹. First, when the axons reach the target, their neurons become dependent on target-derived NGF for their survival. They compete for limiting amounts of NGF, resulting in death of about half of the neurons, thus matching the size of the innervating population to that of the target population. After this cell death period, neurons become independent of NGF for continued survival²¹. NGF also regulates target innervation in two other ways that are more difficult to study because of this transient trophic requirement, but several strategies have circumvented this problem: for example, through use of mice lacking the cell death effector Bax, or application of antibodies after the cell death period. Thus, it has been shown that a target-derived gradient of NGF is required for NGF-responsive axons to invade their target organs, because they stall outside the target in NGF mutant mice that also lack Bax (to prevent cell death)²² or when the gradient is reversed by transgenic autocrine expression of NGF in the neurons²³. Local gradients of NGF within target organs also regulate the degree of axon terminal arborization (Fig. 3b). For example, denervation of

patches of skin causes neighbouring sensory axons to sprout collaterals and re-innervate the denervated terrain. This process is directed by NGF secreted from the denervated tissue, because it is blocked by antibodies to NGF²⁴ and NGF has been shown to act directly on the axons to stimulate sprouting²⁵.

Thus, global and local gradients of NGF (and other growth factors, like BDNF⁴) ensure appropriate target innervation by regulating both target invasion and the formation of an appropriate pattern of terminal arborizations, much as gradients of VEGF ensure target coverage by stimulating the migration of endothelial cells into avascular tissue and regulating the patterning of vessels. In this way, common principles govern terminal vascularization and terminal arborization, although the molecules involved (VEGF versus neurotrophic factors) seem to be distinct.

Molecular guidance cues for developing axons

We now discuss the second parallel in the formation of neural and vascular networks—in this case, involving common molecular cues—that is, how larger vessels and axons follow highly stereotyped anatomical patterns (Fig. 1). How can axons navigate to far away targets over distances exceeding the neuronal cell body diameter by more than a thousand fold? They simplify this task by breaking up their long trajectory into smaller segments bounded by intermediate targets¹. Thus, the complex task of projecting long distances is reduced to the simpler task of navigating a series of short segments—each perhaps a few hundred micrometres long—from one intermediate target to the next. Although endothelial cells do not migrate over distances anywhere near approaching those of the longest axons, they often migrate over trajectories that are similar in length to the shorter segments navigated by axons.

The guidance of axons and endothelial cells is directed by specific cues in the extracellular environment. Over the past decade, considerable progress has been made in understanding axon guidance mechanisms^{1–3}. Guidance cues come in four varieties: attractants and repellents, which may act either at short range (being cell- or matrix-associated) or at longer range (being diffusible). Intermediate targets are often the source of long-range attractive signals that lure axons, and of short- or long-range repellent signals that expel axons that have entered the target, or prevent their entry altogether. In between intermediate targets, axons and vessels are often guided through tissue corridors by attractive cues made by cells along the corridors, and by repulsive signals that prevent them from entering surrounding tissues.

What molecules guide axons? The task of orchestrating the complex wiring of neural circuits—and possibly also of the vascular networks—is largely carried out by a limited number of guidance cues. In the 1990s, four families of axon guidance cues were discovered: the netrins, semaphorins, ephrins and Slits, and their receptors (Fig. 4; and see below). In addition, growth factors (like neurotrophins and hepatocyte growth factor (HGF)) function as axonal attractants and morphogens (Hedgehog, Wnt and BMP proteins) have recently been implicated as both attractive and repulsive cues (reviewed in ref. 26). The responses of axons to these guidance cues show remarkable versatility and plasticity. A single cue may be attractive or repulsive, or regulate axonal branching, depending on the complement of receptors expressed by the responsive neuron or the activity of second messengers in the neuron, and an individual axon may change its response to cues as it develops^{1,2,27}. This plasticity allows an axon to be initially attracted, and then to switch its response such that it becomes repelled by cues in the intermediate target, causing it to move on to the next leg of its trajectory. Examples of this versatility, and the mechanisms that make it possible, are discussed in detail below.

Axon guidance molecules guide blood vessels

We now discuss the mounting evidence for vascular guidance effects of all four families of classical axon guidance molecules. Evidence for

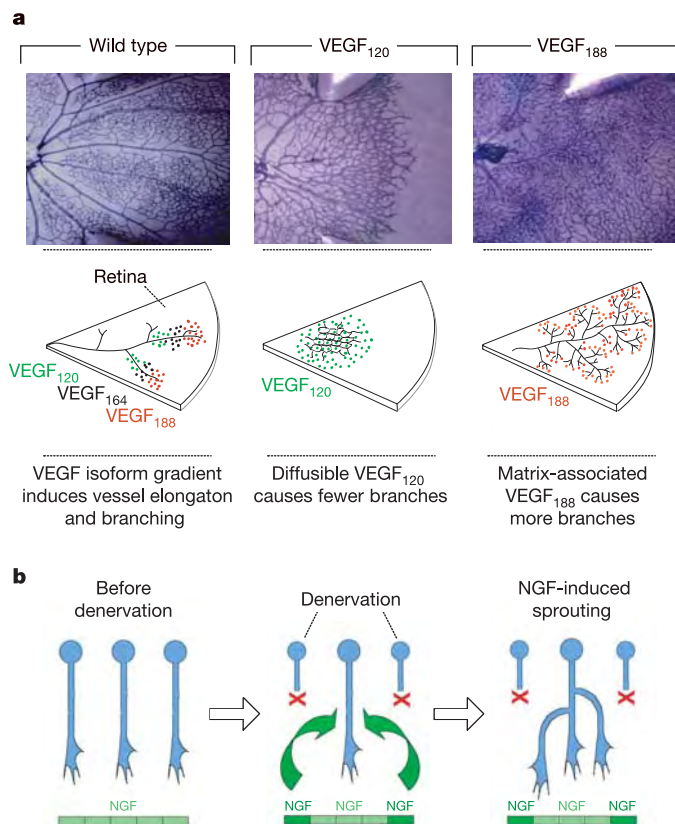


Figure 3 | Growth factor gradients determine vessel and axon branching. **a**, A VEGF isoform gradient determines blood vessel patterning in the neonatal murine retina. The upper panels show the retinal vascular network in wild-type mice (left), VEGF₁₂₀ mice (middle) and VEGF₁₈₈ mice (right), expressing VEGF₁₂₀ or VEGF₁₈₈ alone, respectively (from ref. 20). In wild-type mice, large vessels ramify into smaller branches. Proper elongation and branching of vessels is determined by a spatial gradient of VEGF isoforms, with matrix-associated VEGF₁₈₈ (red) close to the VEGF producer cell, soluble VEGF₁₂₀ (green) diffusing most distantly from the VEGF producer cell, and VEGF₁₆₄ (black) exhibiting an intermediate spatial distribution pattern. In VEGF₁₂₀ mice, the normal VEGF isoform gradient is replaced by a uniform field of ectopic VEGF₁₂₀ expression, causing vessel enlargement at the expense of vessel branching. In VEGF₁₈₈ mice, the VEGF isoform gradient is replaced by local spots of restricted VEGF₁₈₈ expression, inducing supernumerary branching of smaller vessels. **b**, Local gradients of NGF regulate the degree of axon terminal arborization. After denervation, NGF levels are upregulated and cause neighbouring axons to sprout collaterals and re-innervate the denervated terrain.

such effects was first obtained for the semaphorins and ephrins, with more recent evidence implicating Slits and netrins. In each case, we briefly highlight some of their properties in axon guidance and branching, before discussing their functions in vessel guidance.

Netrins and their DCC and Unc5 receptors. In bilateral species, the two sides of the brain must function in a coordinated manner, requiring neurons to connect to the other side by projecting axons across the midline via commissures. Netrins were identified as midline-derived chemoattractants that guide axons to the midline by binding receptors of the DCC (deleted in colorectal carcinoma) family; defects in netrin ligands or their DCC receptors result in defects in reaching and crossing the midline^{1–3,28–30} (Fig. 5a). Netrins are matrix binding, and the extent of their diffusion seems to vary from long range in the spinal cord³⁰ to short range at the optic nerve head³¹ (Fig. 5b). As for many guidance cues, responsiveness to netrins is dynamically regulated during axon navigation. To avoid commissural axon stalling at the midline, where netrin levels are maximal, responses of the axons to the chemoattractant activity of netrin must be silenced³². This is thought to be achieved by Slit proteins, also made by midline cells, which bind roundabout (Robo) receptors, which in turn form a complex with DCC and thereby inactivate netrin's attractant activity³³, allowing the axons to move on (Fig. 5a). Netrins have also been implicated in axon repulsion, an effect mediated by receptors of the Unc5 family^{2,3} acting alone or with DCC receptors³⁴. The evidence suggests that DCC–Unc5 heterodimers mediate repulsion at longer range than Unc5 receptors alone³⁵.

A recent study shows that netrin1 and Unc5b, one of four

mammalian Unc5 receptors, also regulate blood vessel guidance³⁶. *Unc5b* is expressed in endothelial tip cells. Loss of *Unc5b* in mice results in aberrant extension of tip cell filopodia and excessive branching of many vessels. Treatment of cultured endothelial cells or growing vessels *in vivo* with netrin1 induces filopodial retraction. A role for Unc5b in mediating endothelial cell repulsion was confirmed by analysis of the developing intersegmental vessels (ISV) in zebrafish embryos. Pathfinding of these vessels is stereotyped and believed to be genetically programmed by an interaction of attractive and repulsive cues. In control embryos, ISVs sprout from the dorsal aorta to the dorsolateral roof of the neural tube. After knockdown of Unc5b, endothelial cells of ISVs exhibit supernumerary—often ectopically located—filopodial extensions³⁶, and the dorsal trajectory of most ISVs is irregular, with numerous extra branches, and deviates from the normal stereotyped path (Fig. 6a–d). A similar phenotype is observed after knockdown of netrin1a, which is normally expressed in the horizontal myoseptum, located midway along the ISV dorsal migration path. Thus, netrin1a seems to prevent ISVs from entering adjacent somites by activating Unc5b. In mice, the identity of the ligand of Unc5b remains to be determined, as vascular defects have not yet been reported in netrin1 mutants.

A recent *in vitro* study has also suggested a positive role for netrin1 in stimulating distinct endothelial cells; the receptor(s) mediating these effects remain(s) undefined³⁷. In this context, it is of interest that the adenosine A2b receptor, which regulates angiogenesis, can also be activated by netrin binding³⁸, providing a potential route for the positive actions of netrins on these cells. The *in vivo* correlates of the positive effects of netrins seen *in vitro* remain to be defined.

Semaphorins and their neuropilin and plexin receptors. Semaphorins are guidance signals that are secreted and capable of long range diffusion (class 3) but may, in some contexts, have restricted diffusion, or are membrane-bound and function as short range guidance cues^{1–3,39,40}. Semaphorins are best known as repellents, but semaphorin 3A (Sema3A) can also function as a chemoattractant, depending on the intracellular level of cyclic nucleotides²⁷. Semaphorins signal through multimeric receptor complexes: membrane-bound semaphorins bind plexins, whereas secreted class 3 semaphorins bind neuropilins, which function as non-signalling co-receptors with plexins^{2,3,41–44} (Fig. 4a, b). An exception to this rule is the secreted Sema3E, which binds plexinD1 (Plxnd1) directly⁴⁵. Furthermore, the membrane-anchored Sema7A stimulates axon extension by activating integrins⁴⁶. Genetic studies have implicated semaphorins in a wide variety of neural wiring processes (Fig. 5b). Insufficient repulsion by semaphorins results in axon projection defects such as defasciculation, overshooting, aberrant trajectories, misrouting and ectopic termination (see refs 3, 44 and references therein). Semaphorins also direct pruning of pre-established axon branches⁴⁷.

Semaphorins and their receptors also regulate vessel guidance and branching. Endothelial cells express various neuropilin and plexin receptors^{11,48–51}, and Sema3A inhibits formation of endothelial lamellipodia and vessels^{48,51–53}. In mice and zebrafish lacking Plxnd1, ISVs fail to select the appropriate branching site along the dorsal aorta and do not track close to the antero-posterior somite boundaries^{45,50,51} (Fig. 6a, b). Knockdown of Sema3a1 and Sema3a2 in zebrafish largely phenocopied the Plxnd1 knockdown phenotype⁵¹, suggesting that vascular misguidance may result from disrupted Sema3A/Plxnd1-mediated repulsion. ISVs express Plxnd1 and navigate through the intersomitic corridors, which express Sema3a1 and Sema3a2 within their deeper layers, thereby preventing the ISVs from entering the somites. In zebrafish, Sema3A homologues presumably activate Plxnd1 by binding a neuropilin; however, it has not yet been examined whether they can bind Plxnd1 directly. In mice, the Plxnd1 mutant phenotype may reflect primarily loss of repulsion by Sema3E, which binds Plxnd1 directly rather than via a neuropilin⁴⁵ (Fig. 4c), although evidence for an involvement of Sema3A, functioning to inhibit integrin function, has also been obtained⁵³.

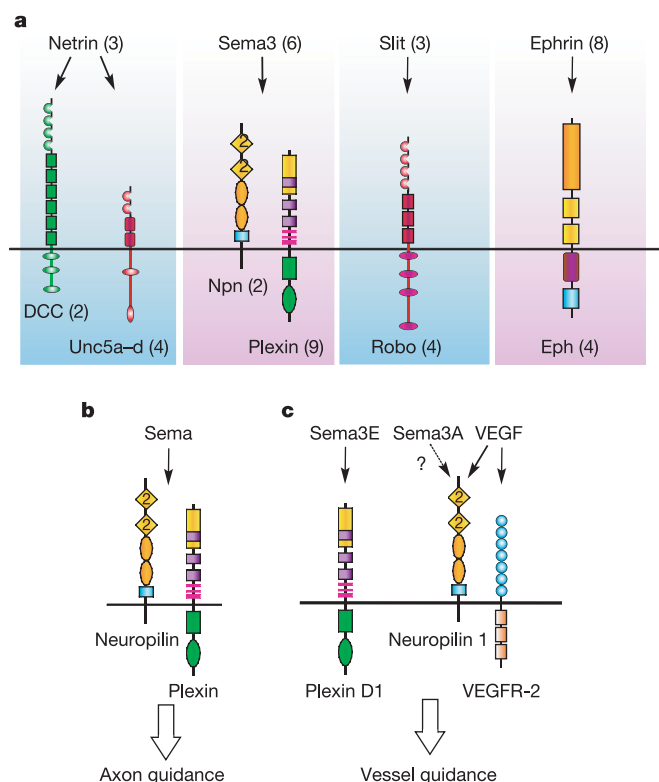


Figure 4 | Principal axon guidance cues and their receptors. **a**, Schematic representation of the four major classes of axon guidance ligands and their receptors; the numbers in brackets refer to the number of known molecules in mammals. **b**, Role of neuropilin–plexin signalling in axon and vessel guidance. Guidance of axon growth cones is dependent on binding of Sema3A to neuropilin1 (Npn1), which induces signalling through its plexin co-receptors. **c**, Guidance of vessels is regulated by binding of Sema3E to Plxnd1 directly, independently of Npn1. In addition, by binding VEGF, Npn1 regulates vessel guidance as a co-receptor of VEGFR-2. Whether binding of Sema3A to Npn1 (and activation of plexins) also contributes significantly to vessel navigation remains to be elucidated.

Neuropilins have also been implicated in vessel patterning—but interestingly, this probably reflects their role in modulating VEGF rather than semaphorin signalling. Neuropilin2 is expressed in veins and lymph vessels, and defects in these vessels are observed in neuropilin2 mutant mice⁵⁴. Neuropilin1 is expressed widely in the

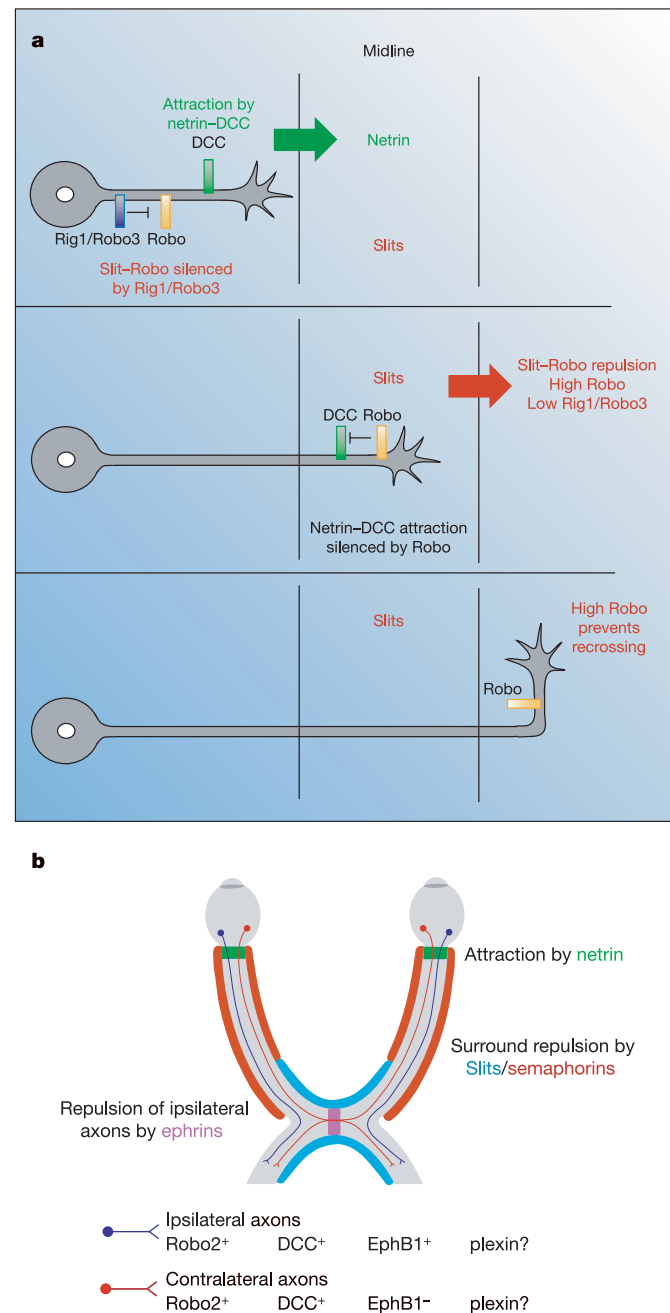


Figure 5 | Mechanisms regulating midline axon guidance. **a**, Roles of netrin-DCC and Slit-Robo signalling in the crossing of commissural axons at the spinal cord midline in mammals. Top panel: before crossing, netrins at the midline attract axons by activating the DCC receptor, whereas Rig1/Robo3 silences Robo, which would otherwise repel axons from entering the midline. Middle panel: at the midline, Robo, in response to Slit, silences DCC, thereby preventing axons from stalling at the midline, and expels the axon from the midline. Bottom panel: once axons have crossed the midline, high Robo levels on the axons prevent them from recrossing the midline. (Adapted from ref. 2.) **b**, Navigation of retinal axons at the optic chiasm. Retinal axons are attracted by netrins (netrin1), present in the optic nerve head, which allows them to leave the eye. Surround repulsion by Slits (Slit1 and Slit2) and semaphorins (Sema5A) prevents RGC axons from straying from their path, whereas repulsive ephrinB ligands (ephrinB2) prevent EphB-expressing ipsilateral axons from crossing the midline. (Adapted from ref. 100.)

developing vasculature, and mice or zebrafish lacking (endothelial) neuropilin1 or both neuropilins exhibit branching and remodelling defects, improper routing and connections, and ectopic termination of vessels^{55–57}. Because neuropilins are receptors for class 3 semaphorins, these results might be interpreted as reflecting loss of semaphorin signalling. However, neuropilins are also receptors for specific VEGF isoforms (VEGF₁₆₅) and modulate the activity of VEGF receptors¹¹; moreover, VEGF₁₆₅ competes with Sema3A for binding to neuropilins⁴⁸ (Fig. 4c). That the vascular role of neuropilin1 may reflect loss of VEGF rather than Sema3A signalling is suggested by findings that mice expressing a variant neuropilin1 only capable of binding VEGF, but not semaphorins, do not exhibit vascular defects⁵⁷. This suggests that a major role for the neuropilins in vascular patterning is as VEGF co-receptors; whether they also mediate semaphorin signals or whether semaphorins signal principally via direct binding to plexins in endothelial cells remains to be explored. Beyond these details, the underlying logic behind the curious duality of neuropilins as simultaneous semaphorin and VEGF receptors also remains to be elucidated.

Slits and their Robo receptors. Members of the Robo receptor family^{58,59} bind Slits (Fig. 4a), which were identified simultaneously as repellents for some axons^{60–62} and as stimulators of branching and elongation of other axons⁶³—reiterating the theme that guidance cues often have dual roles. Secreted Slits function as long- or shorter-range guidance cues. In flies or nematodes lacking Slit, axons that normally never cross the midline now do so, whereas other axons that normally cross the midline only once cross it several times; thus, Slit prevents ipsilaterally projecting neurons from crossing the midline and contralaterally projecting neurons from re-crossing. Mammals have three Slit genes, all of which are expressed by midline cells⁶¹; analysis of a triple *Slit* knockout showed that they also function to expel crossing axons from the midline, a repulsive effect transduced, at least in part, by Robo1 and Robo2 (ref. 64; Fig. 5a). A switch ensures that Slits expel crossing axons only after and not before they cross the midline. There seem to be two components to this switch. First, expression of Robo receptors is low before midline crossing, and upregulated after crossing^{58,64}. In *Drosophila*, Robo expression before reaching the midline is repressed by the regulatory protein Commissureless (Comm)⁶⁵, which keeps Robo receptors intracellularly away from the axonal surface^{66,67}. Upon crossing, this repressive action of Comm is lost, so that surface expression of Robo and, concomitantly, Slit sensitivity is upregulated. A second component recently discovered in mammals involves the Robo family member Robo3 (also known as Rig1), which appears to be an anti-Robo: it is highly expressed before axons cross the midline and functions to prevent premature activation of Slit-Robo repulsion before midline crossing by silencing Robo1 (ref. 68; Fig. 5a). Thus, in mice or humans with mutations in Robo3, axons fail to cross the midline^{68,69}, apparently because of premature activation of Robo1 signalling⁶⁸. By keeping Robo expression low and silenced before crossing, both components of the switch ensure that axons cross the midline. Upregulation of Robo receptors, and downregulation of Robo3, ensure that Slit-mediated repulsion of axons initiates only after midline crossing (Fig. 5a). Together with the silencing of midline attraction described above, this mechanism ensures that axons leave the midline efficiently. Slit-Robo signalling also controls many other guidance events. For example, in *Drosophila*, a combinatorial code of Robo receptors controls the lateral positions of commissural axons after they have crossed the midline and turned longitudinally^{70,71}. Slits also guide ipsilateral and contralateral axons through the optic chiasm by providing a repulsive corridor⁷² (Fig. 5b), and are implicated in dendritic guidance⁷³.

Recently, a vascular-specific Robo homologue, Robo4, has been identified⁷⁴. *In vitro*, Robo4 bound Slit2 in one study⁷⁴, although another failed to detect such binding⁷⁵. Slit2 can repel endothelial cells *in vitro*, and Robo4 may mediate this effect⁷⁴. Intriguingly, another study reported that soluble Robo4 inhibited angiogenesis,

but none of the Slits was found to bind this soluble protein⁷⁵. Further studies will help to determine the extent to which Robo4 mediates the effects of Slit on endothelial cells *in vitro*. A Robo4 knockdown study in zebrafish showed that some Robo4-expressing ISVs failed to sprout from the aorta or arrested midway through their dorsal migration path, whereas others deviated from their normal dorsal trajectory⁷⁶. It remains to be determined whether Robo4 mediates attractive or repulsive signals. Indeed, the impaired sprouting and dorsal extension of the ISVs in Robo4 knockdown embryos might be interpreted as an inability to respond to a chemoattractant signal, but misrouting of the ISVs might also be explained by their unresponsiveness to repulsive signals alongside their normal path. Thus, additional studies on Slits and Robos are required to determine how they contribute to vascular development *in vivo*. Slit–Robo interactions have also been implicated in establishing the non-stereotyped, chaotic vascular network in a tumour model⁷⁷, but the generality of this observation to other tumours remains to be explored.

Ephrins and their Eph receptors. Another principal class of short-range axon guidance molecules is the Eph receptor tyrosine kinases and their ephrin ligands⁷⁸ (Fig. 4a). The 13 Eph receptors in mammals are categorized into A (EphA1–8) and B (EphB1–4 and EphB6) subfamilies. The eight ephrin ligands comprise ephrinA1–5, which are tethered to the membrane via a glycosyl-phosphatidylinositol anchor, and ephrinB1–3, which contain transmembrane and cytoplasmic regions. EphrinA ligands bind EphA receptors, and ephrinB ligands bind EphB receptors; only a modest degree of cross-reactivity between the families has been observed; for example, EphA4 binds some B class ephrins. Eph receptors and ephrins initiate bidirectional signalling in cells expressing Eph receptors (forward signalling) or ephrinB ligands (reverse signalling).

Ephrins were first identified as repellent axon guidance molecules through studies on topographic retinotectal projections^{79,80}, and subsequently have been implicated as both negative and positive cues in other wiring processes: axon guidance at the midline, dendritic spine formation, formation of segregated maps, guidance of neuronal cells, and synaptic plasticity⁷⁸. We illustrate the functions of ephrins by reference to their roles in retinal axon guidance. To establish binocular vision, retinal ganglion cell (RGC) axons from both eyes need to converge on the same target area of the brain. The optic chiasm functions as a midline intermediate target where RGCs choose to project their axons to the ipsilateral or contralateral side of the brain. EphrinB2 is expressed in the chiasm and repels axons of ipsilaterally projecting RGCs^{81,82} (Fig. 5b). Consistent with this, EphB1, a receptor for ephrinB2, is found exclusively in retinal regions giving rise to the ipsilateral projection, and loss of EphB1 reduces

ipsilateral projections⁸². Ephrins also regulate crossing of other axons at the midline^{83,84}.

Ephrins also determine the topographic projection of RGCs to the midbrain superior colliculus in mammals. RGC axons from the nasal retina with low EphA density project to the posterior superior colliculus, where they encounter higher levels of ephrinA repellents, whereas RGC axons from the temporal retina expressing a high density of EphA receptors project to the anterior superior colliculus, with low expression of ephrinA repellents^{78–80}. Relative rather than absolute levels of repellent gradients appear to be important^{85,86}. Indeed, growth of RGC axons is promoted at low ephrinA concentrations in the anterior superior colliculus, but then inhibited at high ephrinA2 concentrations in the posterior superior colliculus, thus helping growth arrest at a site along the anterior–posterior axis of the superior colliculus where both growth-promoting and repellent signals are balanced⁸⁷. The location of this neutral point is determined both by the anterior–posterior ephrinA gradient in the superior colliculus and by the naso-temporal gradient of EphA in the retina.

Eph–ephrin signals also control vascular development⁸⁸. Some of these guidance molecules were among the first factors found to be expressed selectively in either arteries or veins. Historically, haemodynamic pressure differences were presumed to regulate the differentiation of high-pressure vessels into arteries and low-pressure vessels into veins. Expression analysis and loss-of-function studies in mice indicated, however, that EphB4 and ephrinB2 are expressed in developing veins and arteries, respectively, and are critical for their maintenance^{12,13,89}. These studies indicated that repulsive ephrinB2–EphB4 signalling—both forward and reverse—may prevent intermixing of venous and arterial endothelial cells, secure assembly of ‘like’ endothelial cells and demarcate arterial–venous cell boundaries^{90–92} (Fig. 6e)—similar to how ephrins sort neural cells during segmentation of the vertebrate hindbrain into rhombomeres.

Ephrins also regulate vessel guidance and morphogenesis. Repulsive ephrin–Eph signals provide short-range guidance cues for vessels to navigate through tissue boundaries. For instance, ephrinB2 repels EphB3/EphB4-expressing ISVs from entering somites^{12,13,93} (Fig. 5a, b). But the role of ephrin/Ephs may not be so simple—indeed, ephrin–Eph interactions may also provide attractive cues and induce capillary sprouting in other contexts. For instance, juxtacrine expression of ephrinB ligands and EphBs on adjacent endothelial cells or smooth muscle cells in the same vessel may provide bidirectional signals for establishing contact-dependent communication, and promote vessel assembly, sprouting and maturation¹³. EphrinA ligands may also function as positive regulators of vascular morphogenesis. In the adult, ephrinA1, expressed on tumour cells, has been

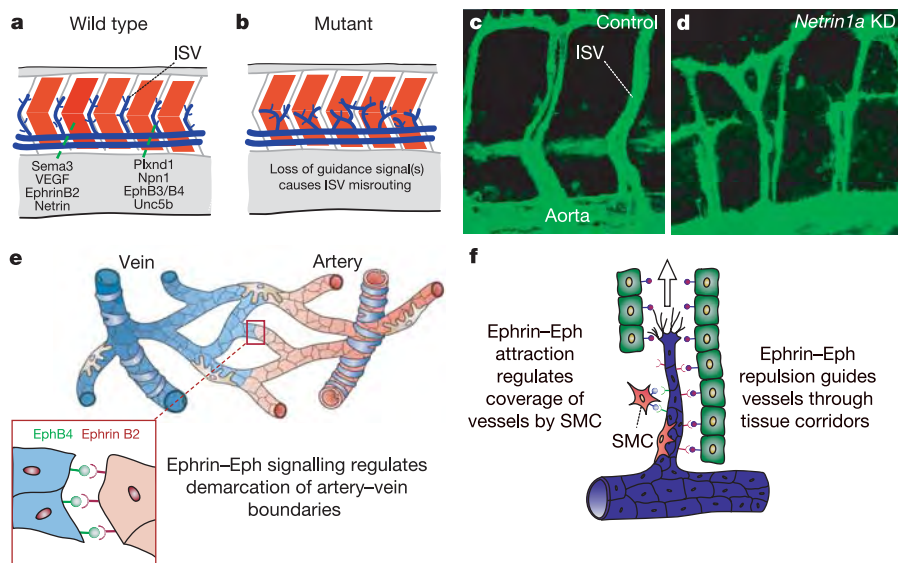


Figure 6 | Role of guidance signals in intersomitic vessel guidance. **a**, Schematic representation of the zebrafish embryo trunk, showing the somites (red) producing the indicated guidance cues and ISVs (blue) producing the indicated receptors. **b**, In the absence of these guidance cues, the ISVs are misrouted. **c**, Stereotyped pattern of ISVs in zebrafish embryos (the endothelial cells express an eGFP transgene). **d**, After knockdown (KD) of *netrin1b*, the ISVs are misguided. **e**, Artery, ramifying into capillaries, which drain into veins. Repulsion between EphB4-expressing venous (blue) and ephrinB2-expressing arterial (red) endothelial cells prevents intermixing of arteries with veins. **f**, Ephrin/Eph family members also regulate other aspects of vessel guidance and morphogenesis, such as recruitment of smooth muscle cells (SMC) towards endothelial cell channels. In addition, repulsive ephrin–Eph signals provide short range guidance cues for vessels to navigate through tissue boundaries.

implicated in guiding EphA2-expressing vessels to the tumour cells, as ephrinA1 can stimulate angiogenesis⁹⁴ and EphA2-Fc inhibits tumour angiogenesis and growth⁹⁵.

Conclusions and perspectives

Half a millennium ago, Vesalius documented that blood vessels and nerve fibres are highly branched in stereotyped patterns. Vessels, which arose later in evolution than nerves, appear to have co-opted the same evolutionarily conserved guidance cues that help axons to navigate to their targets. These findings, as well as the realization that vascular and neural morphogenesis shares many more processes than originally thought⁹⁶, has opened new research avenues and has also raised numerous intriguing questions. First, a variety of distinct growth factors regulate axon terminal arborization in a neuron- and tissue-specific way. Will there be tissue-specific regulators of angiogenic sprouting as well? EG-VEGF, an angiogenic mitogen selective for endocrine gland endothelial cells⁹⁷, provides one example, but it is unclear whether it is an exception or the rule. Second, will other factors more recently implicated in axon guidance—particularly morphogens such as Hedgehogs, BMPs and Wnts—also be found to regulate vessel morphogenesis? Evidence for such involvement is already provided by the recent finding that proper retinal vascularization requires activation of the Wnt receptor frizzled 4 by norrin, which is impaired in Norrie's disease and familial exudative vitreoretinopathy⁹⁸. Members of the fibroblast growth factor family also remain strong candidates for regulating both processes^{1–3,5}. Third, pruning of small vessels and axon terminal arborizations can result from a reduction in positive branching signals (like VEGF and NGF), but in the case of axons can also be triggered by semaphorins⁴⁷—will repellents also act as 'pruning signals' for vessels? Fourth, silencing of a repellent receptor allows an axon to navigate to its target but, once passed by, activation of the receptor will ensure that the axon does not backtrack—does such sophisticated regulation of responsiveness also allow vessels to take turns, cross boundaries and reach their targets? Finally, axon guidance cues guide vessels, but do vessel guidance cues also guide axons? The reports that VEGF modulates growth cone movements⁴⁸, regulates neuronal patterning⁹⁹ and is critical for maintenance of neuronal circuitry justify further investigation of possible axon guidance effects of this canonical angiogenic factor. Finally, discovering how and which signals guide vessels and nerves might also offer novel therapeutic opportunities. Indeed, the evidence that blocking a guidance molecule may impair tumour angiogenesis in an animal model already provides a first glimpse of this therapeutic potential⁷⁷, with many more likely to follow.

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Acknowledgements We regret that, owing to space limitations, we have been unable to refer to all of the primary literature and had to rely instead, in many instances, on reviews. We thank T. Jessell and R. Watts for comments on the manuscript. P.C. is supported by grants from the FWO, the European Union and the Concerted Research Activities of Belgium.

Author Information Reprints and permissions information is available at npg.nature.com/reprintsandpermissions. The authors declare competing financial interests: details accompany the paper on www.nature.com/nature. Correspondence and requests for materials should be addressed to P.C. (peter.carmeliet@med.kuleuven.be) or M.T.-L. (marctil@gene.com).

Insight into the 2004 Sumatra–Andaman earthquake from GPS measurements in southeast Asia

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Data collected at ~60 Global Positioning System (GPS) sites in southeast Asia show the crustal deformation caused by the 26 December 2004 Sumatra–Andaman earthquake at an unprecedented large scale. Small but significant co-seismic jumps are clearly detected more than 3,000 km from the earthquake epicentre. The nearest sites, still more than 400 km away, show displacements of 10 cm or more. Here we show that the rupture plane for this earthquake must have been at least 1,000 km long and that non-homogeneous slip is required to fit the large displacement gradients revealed by the GPS measurements. Our kinematic analysis of the GPS recordings indicates that the centroid of released deformation is located at least 200 km north of the seismological epicentre. It also provides evidence that the rupture propagated northward sufficiently fast for stations in northern Thailand to have reached their final positions less than 10 min after the earthquake, hence ruling out the hypothesis of a silent slow aseismic rupture.

The 26 December 2004 Sumatra–Andaman megathrust earthquake is associated with the continuing subduction process along the Sumatran trench. However, in this particular region, the subducting plate is neither entirely Australia nor entirely India. Therefore it is quite difficult to determine the exact relative motion on the subduction zone. According to the latest geodetic determination of plate tectonics in southeast Asia, the relative motion between Australia and Sundaland is $5 \pm 0.3 \text{ cm yr}^{-1}$ oriented $8 \pm 2^\circ \text{N}$ at the northern tip of Sumatra^{1,2}. Relative to the Indian plate, the motion has a slightly different azimuth (20°N) and a lower velocity of 4 cm yr^{-1} (ref. 3). Previous GPS measurements in this area showed accumulation of elastic deformation in the overriding plate (Sundaland) owing to the locking of the subduction interface⁴. Dip angle and precise locking depths were difficult to estimate accurately, but the extent of residual deformation in the Malaysian peninsula and southern Thailand pointed towards a very large coupling zone. The 26 December 2004 earthquake corresponds to the elastic rebound of this large region. From initial seismological data and tsunami observations it was not clear whether the seismic rupture was confined to a length of ~450 km or continued 500 km to the north in either a seismic mode or in a slow (and ‘silent’) mode. Recent, new GPS data shared in the framework of the EU–ASEAN ‘South-East Asia: Mastering Environmental Research Using Geodetic Space Techniques’ (SEAMERGES) project enable us to quantify the widespread surface deformation and hence to determine the size of the rupture.

GPS-observed co-seismic deformation

The GPS observations used in this study provide a data set that is unique because it provides dense coverage of the surface displacements at intermediate and large scale. Publicly available GPS data in

this region (from the International GPS Service) are limited to only 3 stations at large distances from the earthquake. Our (SEAMERGES) GPS network comprises 49 continuously operating stations in Indonesia (6), Malaysia (38) and Thailand (5). In addition, data from 7 campaign sites in Thailand, observed in October 2004 and in February 2005, are incorporated in the analysis. Furthermore, the network is extended with 9 regional and 21 global stations of the International GPS Service (IGS). The combined co-seismic displacement field is presented in Fig. 1 (and Supplementary Table 1). Only stations located more than 4,000 km away from the epicentre (for example, KIT3 in Uzbekistan and KARR in Australia) are unaffected by the earthquake. Small, but significant, co-seismic jumps between 5 and 10 mm are detected even at stations more than 3,000 km away from the earthquake epicentre—for example, in southern China (Kunming), continental India (Bangalore, Hyderabad) and eastern Malaysia (Sabah). Even stations at Diego Garcia island in the Indian Ocean and in the Philippines were displaced by more than 5 mm. The nearest sites, more than 400 km away from the epicentre, show very large co-seismic displacements: 27 cm in Phuket, Thailand, 17 cm on Langkawi island, Malaysia, and 15 cm in Sampali, Indonesia.

Overall, the deformation field points inward towards the earthquake epicentre. East–west-trending displacements at mid-latitudes (between 0° and 15°), north–south-trending displacements at higher latitudes (below 0° or above 15°), and absence of significant displacements north (LHAS) and south (BAKO) of the rupture are due to a thrust focal mechanism, aligned with the Sumatran trench west of the west coast of Sumatra. Large displacements in northern Thailand (8 cm in Bangkok and almost 3 cm in Chiang Mai) imply a rupture extending far north into the Andaman Sea, in agreement with the distribution of aftershocks. On the other hand, the very strong increase of displacements detected along the Malaysian

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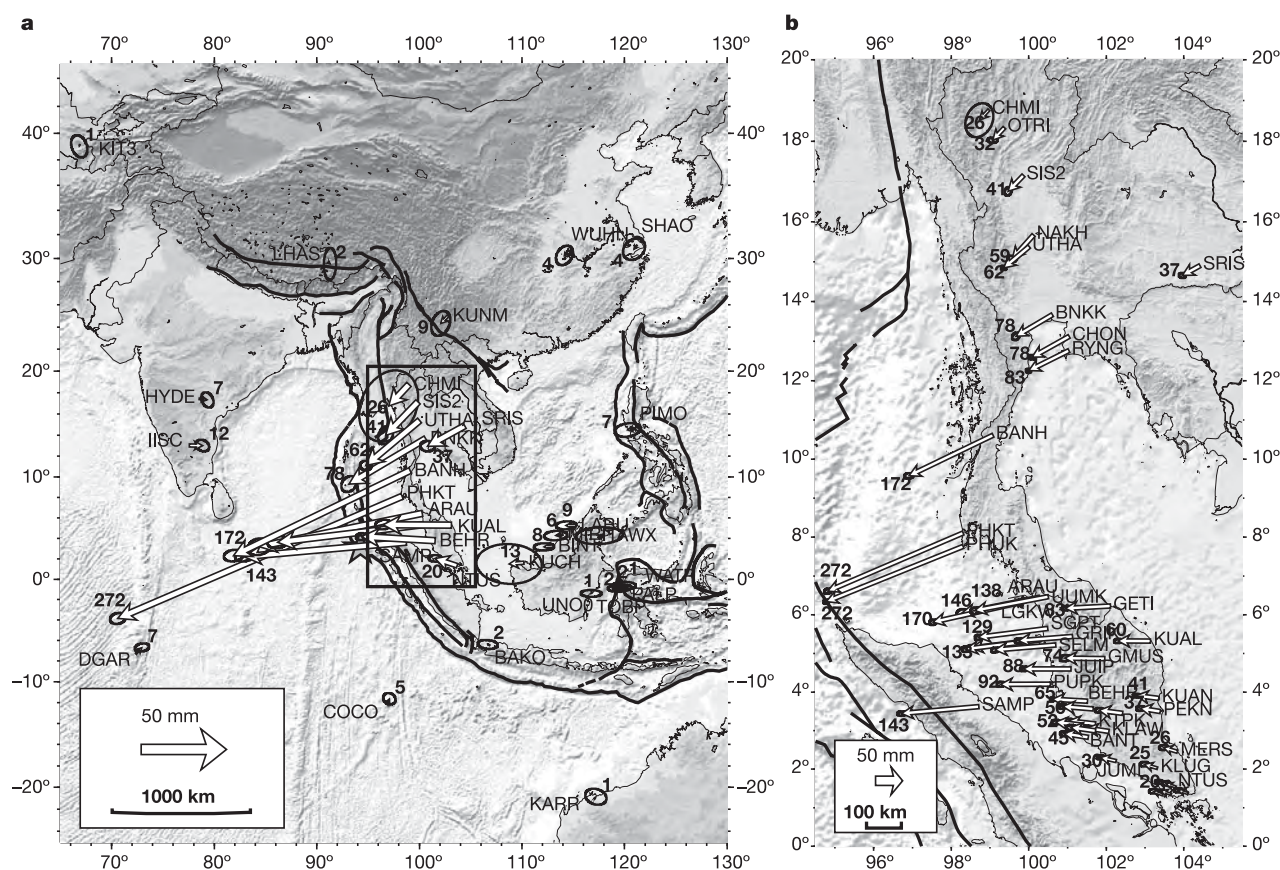


Figure 1 | Co-seismic displacement field derived from GPS observations at more than 60 sites. Panel **a** shows a large scale overview from a low-density subset. Panel **b** provides more detail, zooming in on a smaller area (rectangular box in **a**). Note the high-density sub-network on the Malaysian peninsular. Bold numbers next to arrow heads give the displacement in mm.

Ellipses depict the 90% confidence level. Thin black lines depict major faults⁸. The USGS earthquake epicentre location is portrayed by the star symbol, near bottom left of box. ETOPO-5 and GTOPO-30 Digital Elevation Models were used to generate the background topography and bathymetry.

peninsula (2 cm in Singapore, 17 cm in Langkawi island) suggests a limited amount of slip on the southern section of the fault.

Elastic co-seismic modelling

Observed surface displacements are modelled using Okada's formulation of a dislocation buried in an infinite elastic half-space⁵. A first model (model A) was constructed using the USGS⁶ and CMT⁷ parameters: localization, depth, focal mechanism and magnitude.

To match these parameters, we assume a rectangular dislocation plane of 450 km length and 145 km width, dipping with an angle of 8° and emerging at the surface roughly along the trench. Assuming a rigidity coefficient of 4×10^{11} GPa, a uniform slip of 12 m perpendicular to the trench direction gives a total seismic moment of 3×10^{22} N m and a moment magnitude $M_w = 9.0$, in agreement with the CMT value. With an average misfit of 27 mm this model matches well the observed deformation in northern Sumatra

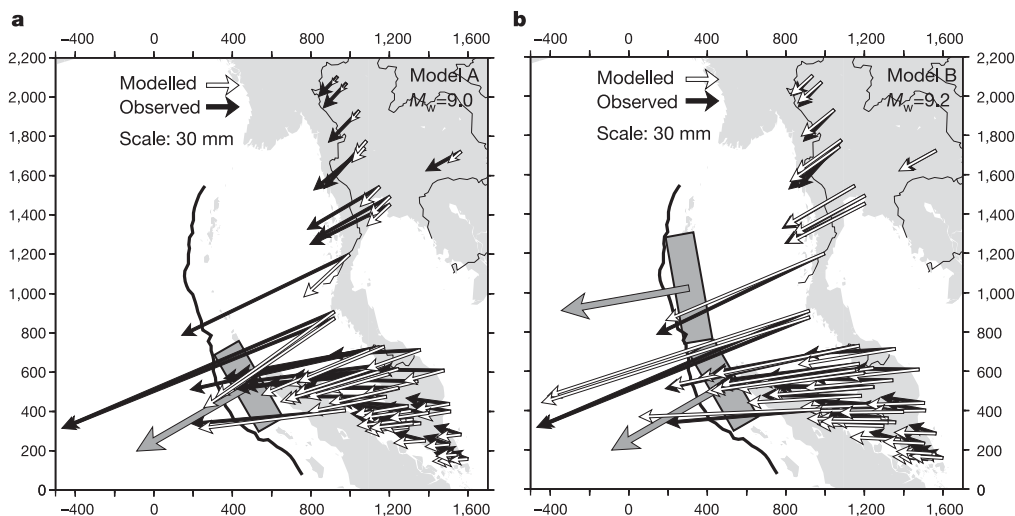


Figure 2 | Elastic modelling of co-seismic deformation. GPS results (black arrows) and model predictions (white arrows) are shown. Grey rectangles depict models for rupture planes buried at depth (see text for details). Grey arrows show the modelled direction and amount of slip. Model A (panel **a**) uses a 450-km-long rupture and model B (panel **b**) uses a 1,000-km-long rupture, curved along the trench in two segments. x and y axes show distance (km) in a UTM (Universal Transverse Mercator) projection.

(SAMP) and the Malaysian peninsula, but fails to predict the large deformation observed in the northern part of the network (Fig. 2). Only half of the observed deformation (15 cm instead of 27 cm) is predicted at Phuket island (PHKT). Only one-third of the observed deformation (3 cm instead of 8 cm) is predicted at Bangkok (BNKK). Finally, insignificant displacements are predicted further north in China and further west in continental India, which is in contradiction with the observations. Therefore, we can conclude that a much longer rupture must be considered.

A second model (not shown) was then constructed by simply prolonging the rupture plane further north to a total length of 1,000 km. All other parameters were kept identical to those of model A. Although this simple model fails to predict the details of the observed deformation field (in particular, the directions in continental Malaysia), it matches better the observed far-field deformation, both in amplitude and direction, with an average misfit reduced to 12 mm. The corresponding seismic moment is increased to 7×10^{22} N m and the magnitude to $M_w = 9.2$. However, it is very clear that such a rupture plane does not follow the trench direction in this area. The trench is curved to an almost north–south azimuth above a latitude of 5° N. Therefore, we constructed a third model (model B) by simply cutting the previous plane into two planes: the southernmost one with a length of 450 km and the original strike of 330° , and the second one with a length of 550 km and striking 350° . The total length and the slip being identical to those of the previous model, the seismic moment and the magnitude remained unchanged. The better alignment with the trench leads to a better

fit with the observed deformation (the average misfit is now reduced to 10 mm), mostly in the predicted deformation direction, as expected (Fig. 2). This is true in particular in the central part of the network, in northern Malaysia and Thailand. As good as it is, this model still predicts too much deformation in the southern part of the Malaysian peninsula. Here, the observed displacements are consistently smaller (by 1 cm on average) than the predicted ones and point more to the north. Reduction of predicted deformation in south Malaysia can only be achieved by reducing the amount of slip on the southern part of the rupture. In fact, a fourth model (not shown)—in which we impose a reduced slip of only 3 m on the first half of the southern plane, balanced by an increased slip on the second half—yields an even better fit, with an average misfit reduced to 8 mm.

In order to investigate the effect of non-homogeneous slip on the fault, we construct a model with a grid of multiple nodes on which we invert the amount of slip, keeping its direction fixed. The surface fault geometry follows the mapping of ref. 8, the dip angle is fixed to 13° according to USGS determination⁶, and the maximum depth is 50 km. All models providing a good fit require a very small amount of slip on the southernmost part of the rupture (which starts around Simelue island, Indonesia), a very localized patch of very large slip (> 20 m) in front of Phuket, Thailand, and a third patch of slip more spread out further north. All models also require that slip stops

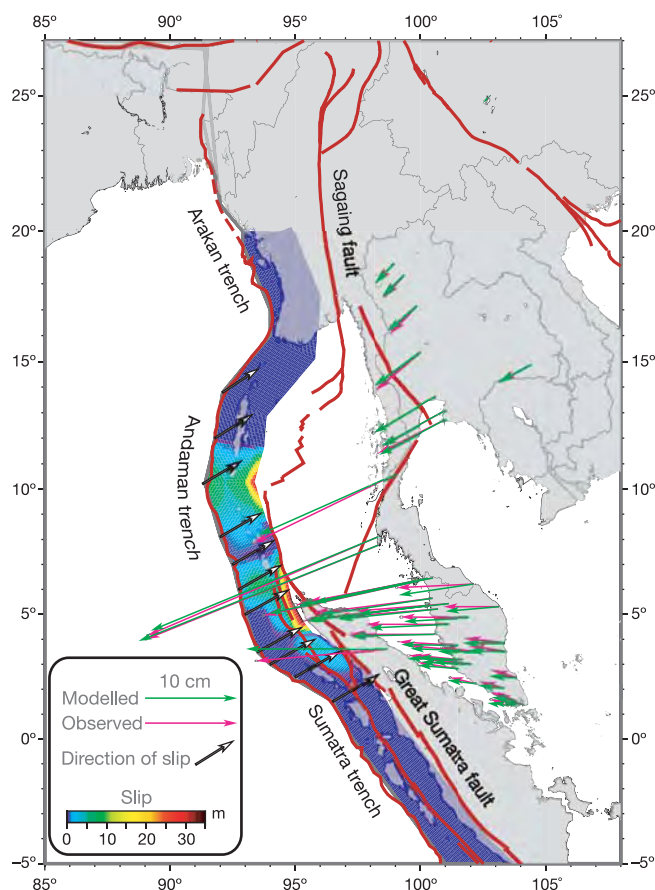


Figure 3 | Best-fit elastic model of the co-seismic displacements using non-homogeneous slip along the rupture plane. Colour code indicates the amount of slip from 0 (dark blue) to 35 m (dark red). Note that the area with significant slip is approximately 1,100 km long. Measured displacements (purple vectors) and modelled deformation (green vectors) are also depicted. Thin red lines depict major faults⁸.

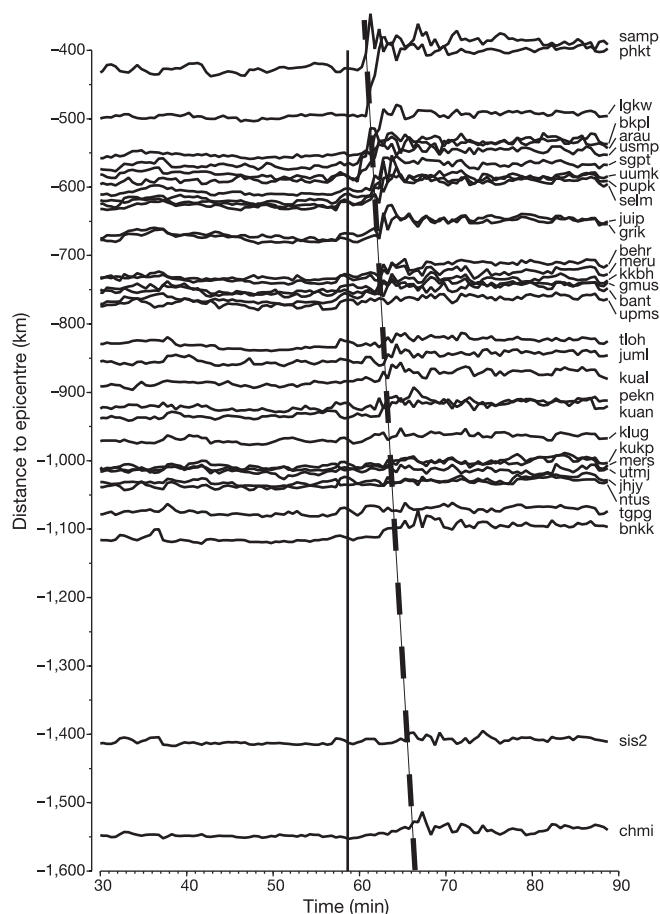


Figure 4 | Kinematic solution of the co-seismic displacements for 33 continuous GPS stations in southeast Asia. Each curve shows the variation in the horizontal position of a given station during 1 h around the time of the earthquake (1 point every 30 s). Curves are sorted by distance to the earthquake epicentre, from 450 km for station SAMP (Medan, northern Sumatra, top) to 1,500 km for station CHMI (Chiang Mai, Thailand, bottom). The vertical full line indicates the time of the earthquake (0 h 59 min) and the second, oblique, dashed line indicates the expected surface wave arrival time assuming a group velocity of 3.6 km s^{-1} .

around 13°–14° latitude, which matches very well the end of the aftershock area⁶ and the crucial changes in the bathymetry of the trench in the area⁹. Also, the patch with hardly any slip between 7° and 8° is a feature that persists throughout all our inversions. The best fitting model (Fig. 3) provides a normalized χ^2 of 2.2 and an average misfit of 4 mm. It requires slip to be moderate near the surface and maximum at the bottom of the fault, possibly like the 1881 Nicobar earthquake¹⁰. Imposing more slip at the surface degrades the fit (χ^2 is increased by 50%), but is still in the range of very plausible solutions: total average misfit is 6 mm. Far-field observed displacements are also matched by our best-fit elastic model. We compute 11 mm (instead of 9 mm) at Kunming, 8 mm at Manila (instead of 7 mm), 13 mm at Bangalore (instead of 12 mm) and 7 mm (as observed) at Hyderabad. Finally, the model predicts displacements of the order of magnitude of 2–5 m near the fault itself, on the Andaman and Nicobar islands. These values will have to be matched with precise GPS surveys conducted there by Indian institutes.

There is a clear trade-off between the maximum slip and the width of the fault or the depth of the fault on which slip is distributed. Obviously, our deformation field lacks the sensitivity of near-field data to fully resolve this issue. Another limitation of our inversion is the assumed constant azimuth of the slip along the rupture plane. Although this azimuth is a reasonable average, there is a slight misalignment with the aftershocks' slip vector direction at the northern termination of the rupture. Finally, the contrast in elastic parameters east of the trench between continental crust in the south and oceanic lithosphere in the north must probably be taken into account. However, the heterogeneity and localization of slip on the southern half of the rupture is in very good agreement with independent determinations from the inversion of short-period seismic body waves (<http://iisee.kenken.go.jp/staff/yagi/eq/Sumatra2004/Sumatra2004.html>). The corresponding seismic moment and magnitude that we compute depends on the assumed rigidity coefficient. Obviously, the GPS data we present here constrain well the length of the rupture, give some insight on the slip $\times$ fault width product, but give no information at all on the rigidity. However, our magnitude of 9.2 matches well the determination of 9.3 inferred using the longest-period normal mode of the Earth over a longer time period than CMT determination¹¹, even if this latter determination has been slightly overestimated because of the neglect of self-gravitational effects.

Kinematic positioning

Although only measurements averaged over 24 h give the ultimate precision of a few millimetres that are expected from GPS, it is also possible to compute each station position on an epoch-by-epoch basis, that is, at the sampling rate of the GPS signal at each station (30 s in this case). GPS stations start to move when surface waves

emitted by the hypocentre hit their location, then within minutes they stabilize to their new position (Fig. 4). Knowing surface wave velocity, it is straightforward to predict 'arrival times' at each station and to match them to the 'jumps' in the time series. GPS records are equivalent to very-low-frequency seismograms, so that they can be used to determine the position of the centroid of deformation at the origin of the bulk of the surface wave emission. In this case, to sort arrival times, we need to move the source of the bulk of the signal almost 200 km north of the USGS-determined epicentre. This corresponds very well to the patch of high slip already determined from our co-seismic static displacements. In addition, it can be seen that stations north of the epicentre reach their final position not only later but also slower than stations in the south. Stations PHKT, SAMP or LGKW have rise times of 1 or 2 min, whereas BNKK and CHMI take up to 5 min to rise. This is due to the slow northward propagation of the rupture along the trench after the initial rupture. Phuket's initial motion (around 2 min 39 s after the earthquake time

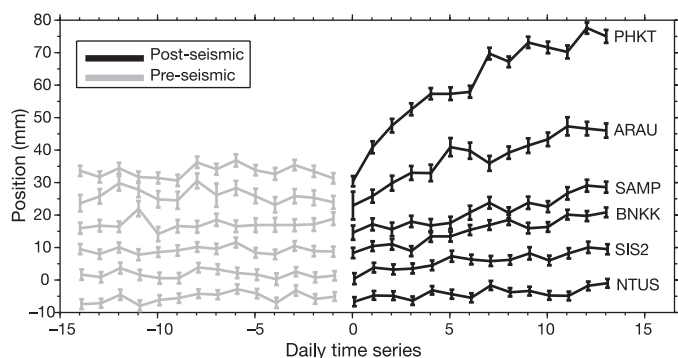


Figure 5 | Pre- and post-seismic position variations derived from GPS observations at selected stations. Daily solutions and 1σ uncertainties are depicted before the earthquake (grey lines) and after the earthquake (black lines). Note the scale is in mm. Initial position is arbitrary, and co-seismic jump is removed.

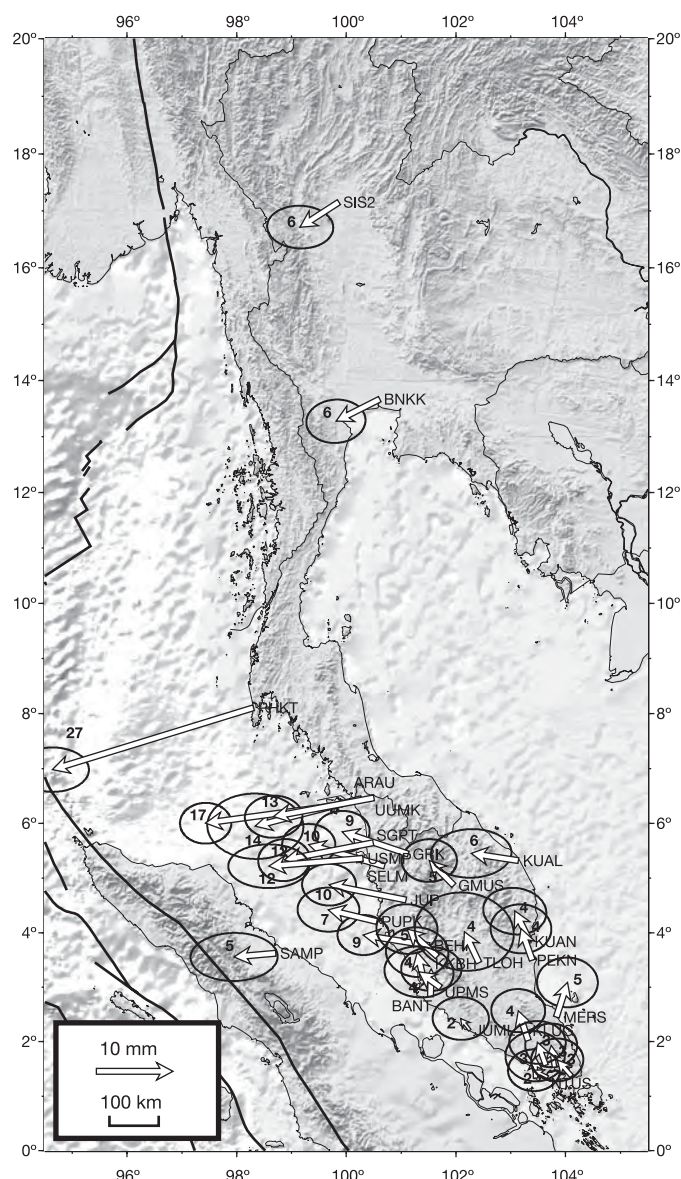


Figure 6 | Five-day post-seismic displacement field. Bold numbers next to arrow heads give the displacement in mm. Ellipses depict the 90% confidence level. Black lines depict major faults⁸. ETOPO-5 and GTOPO-30 Digital Elevation Models were used to generate the background topography and bathymetry.

tag) is towards the south-southwest, in the direction of the point where the rupture started. Three minutes later, the point stops moving and the displacement vector points towards the west-southwest, where the first rupture ended. During this elapsed time, the rupture travelled through a distance of approximately 650 km, yielding a velocity of 3.7 km s^{-1} . The second half of the rupture is slightly smaller (450 km) and is covered in around 4 min. This yields a slower rupture velocity of around 2 km s^{-1} . (A GIF animation is available; see Supplementary Information.)

Given the sampling rate of 30 s of the GPS signal, the uncertainty can be roughly estimated at $\pm 0.5 \text{ km s}^{-1}$. Therefore, it can be concluded that the second part of the rupture is significantly slower than the first part. Nevertheless, the fact that the northernmost stations reached their final position less than 10 min after the earthquake rules out the possibility of a completely silent aseismic rupture (that is, with a period $> 600 \text{ s}$).

Post-seismic deformation

Post-seismic deformation results from a combination of different phenomena, each of which has a characteristic timescale. Deformation occurring over a period of a month represents mostly immediate after-slip, due to either aseismic slip in the poorly consolidated sediment layer overlying the fault or co-seismic slip associated with the aftershock sequences. Deformation occurring over a period of a year might be dominated by poro-elasticity, and thereafter by visco-elastic or plastic flow from low-viscosity shallow earth layers. An increasing amount of evidence indicates that after-slip follows the majority of earthquakes, particularly in subduction zones^{12–14}. Here also, post-seismic motions started right after the co-seismic elastic displacements (Fig. 5). Additional displacements of up to 4 cm over a 15 d period after the earthquake are detected at the nearest station (PHKT). Here, the apparent logarithmic decay suggests aseismic slip in a shallow layer rather than cumulated aftershock-induced slip which should follow the well known hyperbolic trend (Omori's law)¹⁵. The pattern of after-slip cumulated 5 d after the earthquake is also more probably due to aseismic slip (Fig. 6 and Supplementary Table 2). After-slip is maximum at PHKT (3 cm) and in the same direction as co-seismic slip. The along-trench orientation of the observed vectors in south Malaysia is difficult to model with an elastic dislocation. However, the rest of the vectors point to the same patches of high slip that were identified in the co-seismic section. Finally, relative to co-seismic slip, after-slip seems to be proportionally more important in the northern section of the rupture than in the south.

Post-seismic motions continue, and will continue for a long period of time. Fifty days after the earthquake, the island of Phuket had already moved 34 cm, which is 1.25 times the initial co-seismic displacement.

Seismic hazard in southeast Asia

An earthquake on the Sumatran trench was not unexpected. In the Sumatran subduction zone, convergence normal to the trench occurs at $4\text{--}5 \text{ cm yr}^{-1}$. Even near its boundaries, internal deformation of the Sundaland plate is generally small ($< 3 \text{ mm yr}^{-1}$)¹⁶. North Sumatra was a notable exception, as both local and regional GPS networks there detected significant deformation hundreds of kilometres away from the trench^{2,16}. This deformation was interpreted as accumulation of elastic deformation over a wide area, indicating 'high coupling' with subduction. This is in contrast with south Sumatra (south of Enggano island, approximately 3° S) and Java, where inter-seismic deformation is less intense, which is probably related to the variable dip angle and changing obliquity of the subduction. However, because it ruptured only a segment of the subduction zone, the earthquake increased stress on adjacent segments, further south on the Sumatran trench¹⁷, but also further north on the Arakan trench in Myanmar. The M_w 8.7, 28 March earthquake is a first consequence of that. In addition, strain favouring future rupture has probably been

added on the strike-slip system behind the subduction: the Great Sumatran fault in Sumatra and the Sagaing fault in Myanmar. Along-strike additional strain depends on the precise orientation of the rupture: essentially zero if exactly perpendicular to the strike-slip system, it will be increased if oriented north of this azimuth, and decreased if oriented south. In all cases, normal strain will be applied to these faults, favouring unclamping and easing slip. Because historical major earthquakes on these faults have proved that they release strain in a seismic mode^{18,19}, the probability of large earthquakes on these structures in the near future is very high.

Finally, stress transfer has been shown to potentially trigger earthquakes on surrounding faults²⁰. This stress transfer can be instantaneous (Coulomb stress failure) or delayed in time if associated with fluid migration in the brittle crust, and this is particularly true for the unclamping effect²¹. Should this effect play an important role, the foreseen time delay could be of the order of magnitude of a year. Therefore, post-seismic measurements (by GPS stations installed after the earthquake) monitoring the surface deformation for the years to come will provide crucial information on the earthquake mechanism and on possible follow-on scenarios.

METHODS

Static positioning. The data are processed with the GIPSY-OASIS II software, using the (optimized) Precise Point Positioning (PPP) methodology²², including ambiguity resolution for the entire network. For the continuously operating stations, this results in a set of independent daily network solutions, covering a period from 14 d before to 14 d after the earthquake. These daily solutions are subsequently combined into two campaign-like averaged solutions (before and after the earthquake). Next, the pre-earthquake solution is mapped onto the International Terrestrial Reference Frame (ITRF) 2000²³, using a subset of 14 well-determined IGS global reference stations with a smooth positioning history. This is done to establish an undeformed reference solution, which is not affected by episodic jumps in the time series of some of the IGS stations. The post-earthquake solution is then projected on this reference solution using only those stations that are unaffected by co-seismic motions. The co-seismic displacements of the stations in the earthquake region then simply follow from coordinate differences between the two solutions, with a relative (2σ) accuracy of about 2 mm. The GPS campaign data of the 7 additional sites in Thailand are treated in a slightly different way to compensate for the larger time difference between the two campaigns (4 months) and to remove the effects of post-seismic deformation. This is done by removing the long-term rigid plate motions predicted at these locations according to the latest geodetic models¹⁶ and by correcting the total displacements with an estimation of the post-seismic motions based on the results of nearby permanent stations. For the 7 additional Thailand sites a differential horizontal positioning (2σ) accuracy of 6 mm is achieved.

Kinematic positioning. In this approach, absolute station position are computed every 30 s with the GIPSY PPP methodology by using high-rate GPS satellite clock solutions. Each point has a higher uncertainty and is affected by biases which usually cancel out over long periods of measurement. However, the co-seismic signal is clearly detectable in these data: stations within a range of 1,500 km from the earthquake epicentre show displacements up to 3 cm, which is significantly higher than the high-frequency noise of around 1 cm typically obtained in kinematic positioning.

Elastic modelling. Forward models (Fig. 2a, b) are computed with RINGCHN software²⁴. The average misfit of a given model is simply the sum of the residual displacements (difference between observed and modelled displacements) at each station, divided by the number of stations.

Inverse models are computed with DEFNODE software (<http://www.rpi.edu/~mccafr/defnode>; refs 25, 26). The inversion gets the parameters that minimizes the reduced χ^2 statistic: $\chi^2_r = \sqrt{\sum r^2 / s^2} / \text{dof}$, where r is the residual, s is the standard deviation and dof is the degree of freedom. Minimization is performed using the downhill simplex technique.

Received 17 March; accepted 16 June 2005.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements Thanks are extended to the different national agencies (DSMM, RTSD, BAKOSURTANAL) for sharing their regional GPS data in the framework of the SEAMERGES (<http://www.deos.tudelft.nl/seamerges>) project. We also thank M. Hashizume for contributing Thai data collected by Chulalongkorn University in cooperation with the 'Frontier Observational Research System for Global Change' (FRONTIER) project and the Earthquake Research Institute at the University of Tokyo; and we thank the Dutch research programme Integrated Solid Earth Science (ISES), the French Institut National des Sciences de l'Univers (INSU-CNRS) and the French ministry of foreign affairs (MAE) for providing equipment and financial support. Our sympathy is extended to the relatives of the Phuket GPS station operator who is still reported missing after the tsunami disaster. SEAMERGES has been funded by the ASEAN-EU University Network Programme (AUNP). The contents of this paper are the sole responsibility of the authors listed and cannot be regarded as reflecting the position of the European Union.

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Blimp1 is a critical determinant of the germ cell lineage in mice

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Germ cell fate in mice is induced in pluripotent epiblast cells in response to signals from extraembryonic tissues. The specification of approximately 40 founder primordial germ cells and their segregation from somatic neighbours are important events in early development. We have proposed that a critical event during this specification includes repression of a somatic programme that is adopted by neighbouring cells. Here we show that Blimp1 (also known as Prdm1), a known transcriptional repressor, has a critical role in the foundation of the mouse germ cell lineage, as its disruption causes a block early in the process of primordial germ cell formation. Blimp1-deficient mutant embryos form a tight cluster of about 20 primordial germ cell-like cells, which fail to show the characteristic migration, proliferation and consistent repression of homeobox genes that normally accompany specification of primordial germ cells. Furthermore, our genetic lineage-tracing experiments indicate that the Blimp1-positive cells originating from the proximal posterior epiblast cells are indeed the lineage-restricted primordial germ cell precursors.

Primordial germ cells (PGCs) are the source of totipotency, a unique state generated by this lineage. Germ cells have many exceptional properties, including the potential for extensive epigenetic reprogramming of the genome^{1,2}. A detailed understanding of germ cell properties, together with the mechanisms of germ cell specification and their segregation from somatic neighbours, could greatly advance our knowledge of epigenetic mechanisms that regulate cellular differentiation, genome reprogramming and stem cell biology.

Previous studies have shown that germ cell fate in mice is imposed upon pluripotent epiblast cells, which are also the source of all somatic cells^{3–6}. The competence to form PGCs in mice is not an inherited property, but is induced in the proximal epiblast cells by signals from extraembryonic tissues^{5–8}. We have previously shown that germ cell competence results in the upregulation of *fragilis* (also known as interferon-induced transmembrane protein 3 of *Ifitm3*) in the proximal epiblast at embryonic day (E)6.5 (ref. 9). Subsequently, approximately 40 cells acquire PGC fate at about E7.5, as detected by expression of *stella* (also known as developmental pluripotency-associated 3 or *Dppa3*), the earliest known marker of founder PGCs^{9,10}. A key event leading to PGC specification also involves repression of homeobox (*Hox*) genes⁹. Similar repression of the somatic programme is commonly observed in the emerging germ cell lineage in a number of organisms, but the underlying molecular mechanisms involved differ markedly^{11–14}. Here we describe the critical role of Blimp1, a known transcriptional repressor^{15–17}, during PGC specification in mice. We also show that Blimp1-positive cells constitute lineage-restricted PGC precursors much earlier in development than previously thought⁵.

Blimp1 expression marks the origin of PGCs

To identify genes with a key role during specification and segregation of PGCs from their somatic neighbours, we performed differential screen and expression analyses for candidates, including histone methyltransferases, using single-cell complementary DNAs from E7.5 founder PGCs and neighbouring somatic cells⁹. Among the candidates, B-lymphocyte-induced maturation protein-1 (*Blimp1*) showed remarkably specific expression in PGCs but not in somatic cells (Supplementary Fig. 1 and data not shown). *Blimp1* encodes a transcriptional repressor with a SET domain and Krüppel-type zinc-fingers. It drives terminal differentiation of B cells into immunoglobulin-secreting plasma cells^{15,16} by repressing the mature B-cell gene expression programme¹⁷. *Blimp1* is widely expressed during development, including in migrating germ cells¹⁸. We therefore decided to investigate this gene for its role in PGC specification.

We first used *in situ* hybridization to examine the expression of *Blimp1* in late streak stage embryos (E7.25) and observed a strong signal in the posterior-proximal extraembryonic mesoderm, where founder PGCs reside (Fig. 1a), as well as in the visceral endoderm¹⁸. Compared with *fragilis*¹⁹, *Blimp1* expression appears to be more restricted and punctate. Furthermore, we detected 20–25 *Blimp1*-positive cells, and only 2–8 *stella*-positive cells at this stage. Single-cell cDNA analysis confirmed that *Blimp1* expression precedes that of *stella* (see Supplementary Fig. 1a). However, at the early bud stage, *stella* expression was fairly consistently detected in the *Blimp1*-positive cells. These findings led us to reason that PGC specification progresses in the *Blimp1*-positive cells that go on to express *stella*.

Blimp1-positive cells in the early epiblast

Although we initially detected *Blimp1* expression in the visceral

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endoderm (an extraembryonic tissue layer) at E5.5, the earliest expression in the embryo proper was seen at E6.25 (an unexpectedly early stage) in the most proximal layer of the epiblast (Fig. 1b). Notably, these *Blimp1*-positive cells were restricted to only the future posterior side of the shorter embryonic axis (Fig. 1c, d), which has recently been shown to form the primitive streak after dynamic

morphological rearrangement^{20,21}. This highly restricted location is consistent with the expression of other posterior markers²² in *Blimp1*-positive cells at the mid-streak stage, such as *nodal* (*Nodal*), *cripto* (*Cfc1*), *Fgf8*, *brachyury* (*T*) and *Evx1* (refs 20–22 and M.S., unpublished observations). Although *fragilis* is also expressed in the most proximal epiblast in the pre/early streak embryos^{9,19}, it is detected in the entire rim of the proximal epiblast (both anterior and posterior), and in cells that were 3–5 layers deep (see Supplementary Fig. 1b). In contrast, the single layer of *Blimp1*-positive epiblast cells (Fig. 1c, d) is probably in direct contact with the extra-embryonic ectoderm. At the early streak stage, a few cells in the nascent mesodermal region also started to show *Blimp1* expression (Fig. 1d), and in slightly later embryos undergoing

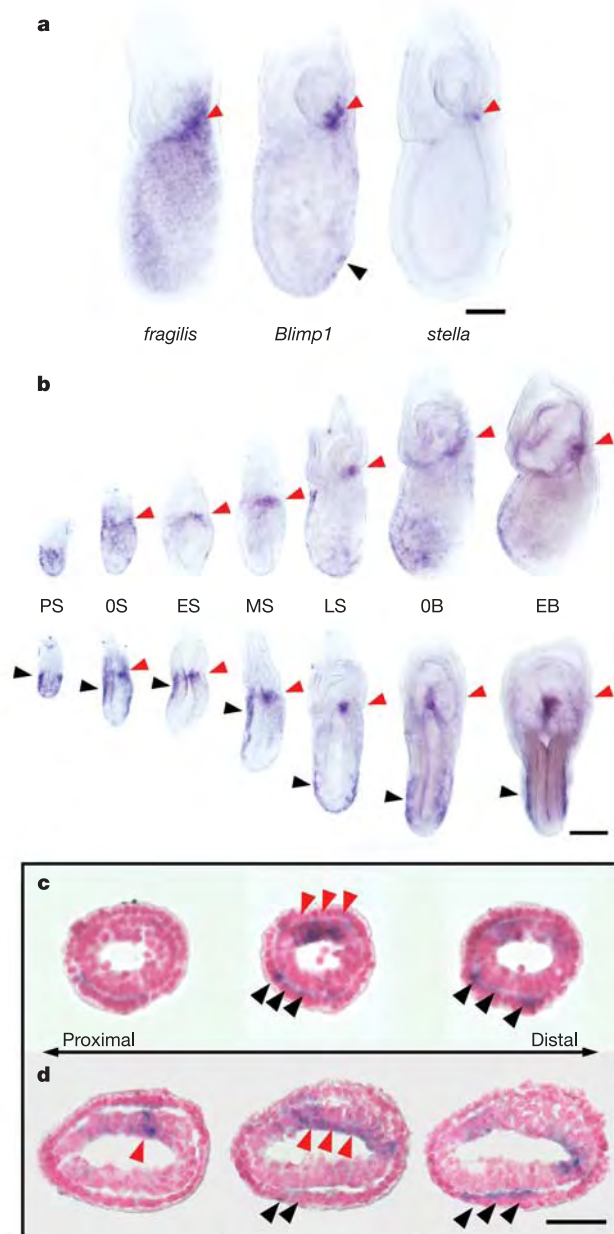


Figure 1 | Expression of *Blimp1* in gastrulating embryos. **a**, *fragilis*, *Blimp1* and *stella* expression at the late streak stage. Anterior is to the left. Red arrowheads indicate the location of founder PGCs and the black arrowhead indicates *Blimp1* expression in the visceral endoderm. Scale bar, 100 μ m. **b**, *Blimp1* expression between pre-streak and early bud stage. Top, lateral view; bottom, posterior view. Scale bar, 200 μ m. PS, pre-streak; OS, no streak; ES, early streak; MS, mid-streak; LS, late streak; OB, no bud; EB, early bud. **c**, **d**, Consecutive 8- μ m sections of the OS (**c**) and ES (**d**) embryos shown in **b**. The left position of the long embryonic axis corresponds to the future anterior (see Fig. 2k). *Blimp1* is detected in the top layer of proximal epiblast cells at one side of the embryo (red arrowheads). Expression in the visceral endoderm (black arrowheads) is primarily on the opposite side. Scale bar in **d**, 100 μ m.

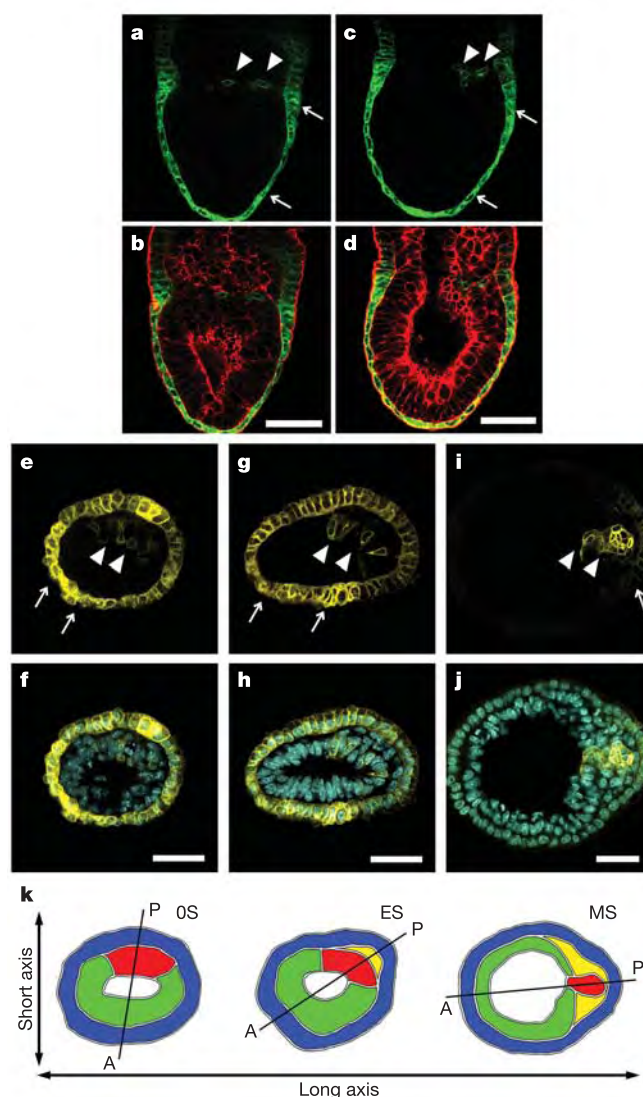


Figure 2 | Origin and allocation of *Blimp1*-mEGFP cells. **a–d**, Lateral views of pre/no streak (**a**, **b**) and early streak (**c**, **d**) embryos stained with phalloidine (red in **b**, **d**). Expression of *Blimp1*-mEGFP is in the most proximal epiblast cells in contact with extra-embryonic ectoderm (white arrowheads in **a** and **c**). **e–j**, Transverse views of pre/no-streak (**e**, **f**), early streak (**g**, **h**) and mid-streak (**i**, **j**) embryos with GFP expression (yellow) in the epiblast (arrowheads) and visceral endoderm (arrows). DAPI (nuclear) staining is shown in cyan. Scale bars, 50 μ m. **k**, Rotation of the embryonic axis and distribution of *Blimp1*-positive cells during gastrulation. *Blimp1*-positive cells (red), *Blimp1*-negative epiblast cells (green), nascent mesoderm (yellow) and visceral endoderm (blue) are shown. A, anterior; P, posterior.

extensive gastrulation movement, the signal was mostly observed in the mesodermal region of the primitive streak (data not shown).

To gain further insight into the origin of the *Blimp1*-positive cells, we used two independent transgenic mouse lines generated using a modified 230-kilobase (kb) bacterial artificial chromosome (BAC) expressing membrane-targeted enhanced green fluorescence protein (mEGFP) under the control of *Blimp1* regulatory elements (see Supplementary Fig. 2). The expression pattern of the reporter transgenes was indistinguishable from *in situ* hybridization analysis (see Supplementary Fig. 2b–d). Serial confocal images of transverse

sections of pre/no-streak stage embryos confirmed the presence of fluorescent cells at one side of the most proximal layer of the epiblast, which is in direct contact with the extra-embryonic ectoderm (Fig. 2a, b, e, f, k). During subsequent development, the number of *Blimp1*-positive cells apparently increased (Fig. 2c, d, g, h, k), and at the mid-streak stage, the fluorescent cells were detected as a cluster at the posterior end of the embryo (Fig. 2i–k). These cells developed a cytoplasmic ‘spot’ in the presumptive Golgi apparatus, which is a characteristic associated with PGCs²³ (Supplementary Fig. 2d and data not shown).

We next determined the numbers of *Blimp1*-positive cells at each stage of development. In pre/no-streak embryos (E6.25), we detected about six fluorescent cells (Fig. 3a), which increased to 16 positive cells at the early streak stage (E6.5), including a few within the nascent mesoderm (Figs 2g and 3a). The relatively abrupt increase in their numbers (Fig. 3a) might be due to a lag period necessary for generating detectable levels of EGFP. These cells form a tight cluster of approximately 20–28 cells at the mid- to late-streak stage (E7.25, Fig. 2i–k), from which tissue non-specific alkaline phosphatase (TNAP)- and stella-positive cells may eventually emerge. In early

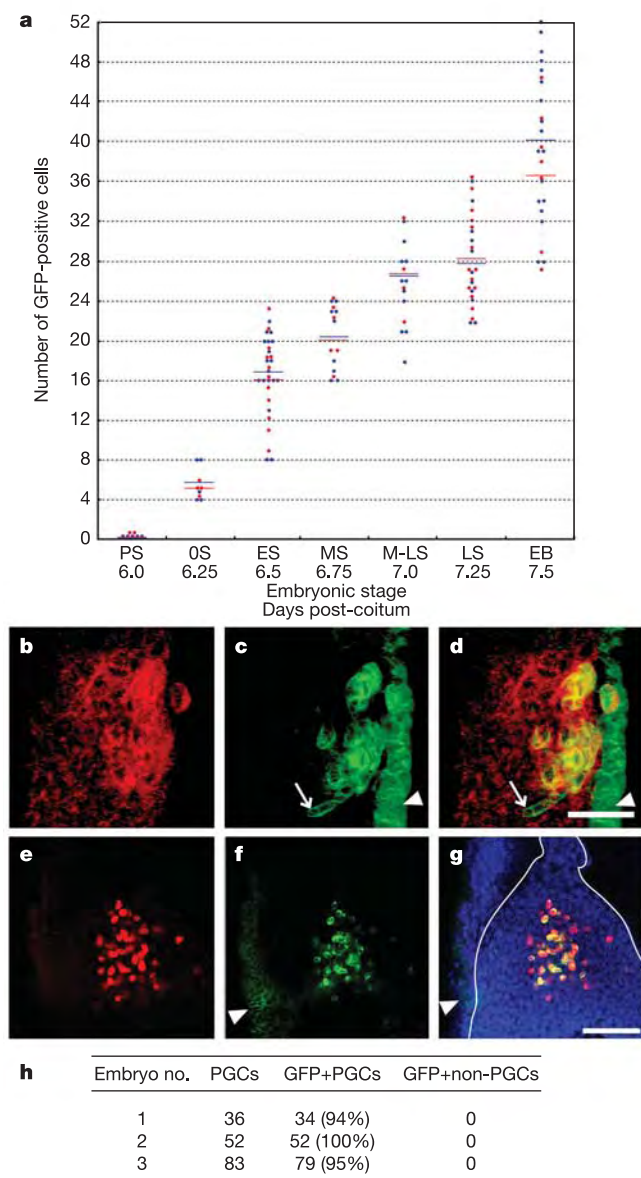


Figure 3 | *Blimp1*-positive cells become TNAP- and stella-positive PGCs. **a**, *Blimp1*-mEGFP cell numbers at different embryonic stages using two transgenic mouse lines (with either 8 copies (blue) or 1 copy (red) of *Blimp1*-mEGFP). **b–d**, Base of the allantois region of early bud stage embryos stained for TNAP (**b**, red) and *Blimp1*-mEGFP (**c**, green). A merged image is shown in **d**. Scale bar, 50 μ m. *Blimp1*-mEGFP cells overlap with TNAP-positive PGCs, with very few exceptions (arrow in **c** and **d**). **e–g**, Posterior view of embryos at early head fold stage stained for stella (**e**, red) and *Blimp1*-mEGFP (**f**, green). A merged image (with DAPI staining in blue) is shown in **g**. Scale bar, 100 μ m. Arrowheads denote visceral endoderm. **h**, Almost all stella-positive cells are also *Blimp1*-mEGFP positive.

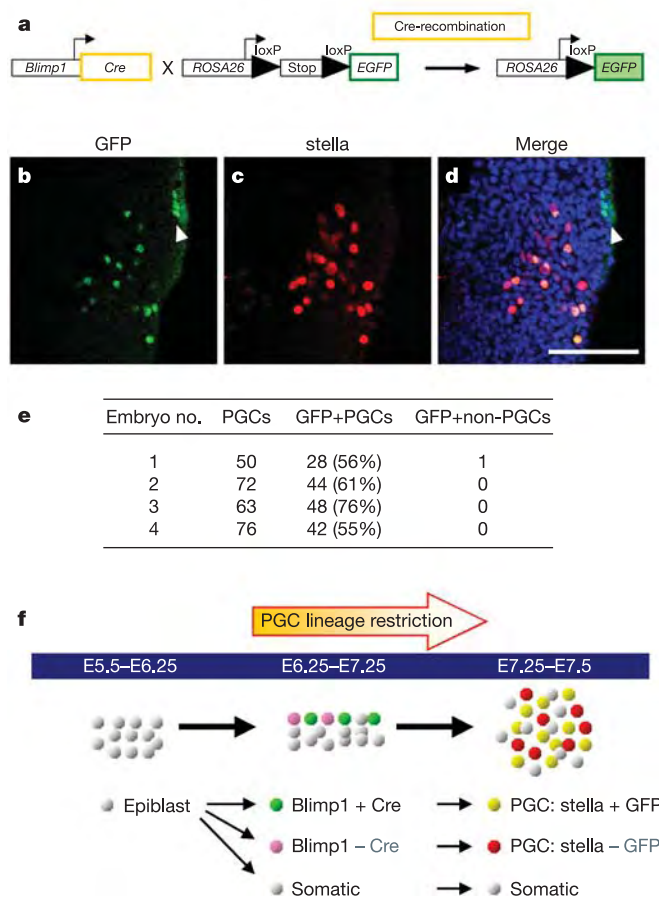


Figure 4 | *Blimp1*-positive cells are lineage-restricted PGCs. **a**, Diagram showing the genetic cross for lineage tracing experiments. **b–d**, Late bud stage embryo (E7.8) stained with anti-GFP (**b**, green) and anti-stella (**c**, red) antibodies. Lateral confocal sections showing the base of the allantois. A merged image (with DAPI staining in blue) is shown in **d**. Scale bar, 100 μ m. All of the GFP-positive cells are stella-positive, except in the visceral endoderm (arrowheads). **e**, PGC cell counts (stella-positive) and the proportion of GFP-positive PGCs. **f**, Cell fates observed in this experiment. *Blimp1*-negative cells (grey) become somatic cells. Cells expressing *Blimp1* and with successful excision of the floxed Stop (green) become stella- and GFP-positive PGCs (yellow). Cells that are *Blimp1*-positive but Cre-negative (pink) later become stella-positive but remain GFP-negative PGCs (red).

bud stage embryos (E7.5) that begin to form the allantois, we observed a cluster of approximately 40 *Blimp1*-positive cells with the cytoplasmic spots characteristic of PGCs (Fig. 3a and Supplementary Fig. 2h–j). We did not detect any *Blimp1*-mEGFP cells in the allantoic mesoderm (the closest somatic relative of PGCs), the yolk sac mesoderm or blood islands. We also note an absence of *Blimp1*-mEGFP-positive cells in mice mutant for bone morphogenetic protein 4 (*Bmp4*; data not shown), which is consistent with a lack of PGCs in these mutants^{7,24}. However, we do not currently know whether there is a relationship between *Bmp4* and *Blimp1* expression in the epiblast.

Blimp1-positive cells are lineage-restricted PGCs

Next, we examined whether the *Blimp1*-positive cells are lineage-restricted to give rise to only PGCs. First, we found that all but a few *Blimp1*-positive cells were co-stained with TNAP (Fig. 3b–d), a

classical marker of founder PGCs, at the early bud stage²³ and also at the mid bud stage (see Supplementary Fig. 3). Furthermore, at the early head fold stage, we found an almost complete overlap between *Blimp1*-mEGFP-positive cells and cells that were co-stained with an anti-stella antibody (Fig. 3e–g), which is a definitive marker of newly established founder PGCs^{9,10,25}. Counts of PGCs in individual embryos showed that 94–100% of cells showed expression of both markers, suggesting that the *Blimp1*-mEGFP-positive cells in the mesoderm are indeed PGCs (Fig. 3h).

To firmly establish that the *Blimp1*-positive cells are lineage-restricted PGCs, we performed a genetic lineage tracing experiment. We created *Blimp1*-Cre-BAC transgenic mice, which we crossed with mice containing a *ROSA26-EGFP*^f reporter²⁶. In doing so, *Blimp1*-Cre-expressing cells and their clonal descendants would become permanently and genetically marked by the onset of GFP expression following Cre-mediated deletion of the Stop sequence in

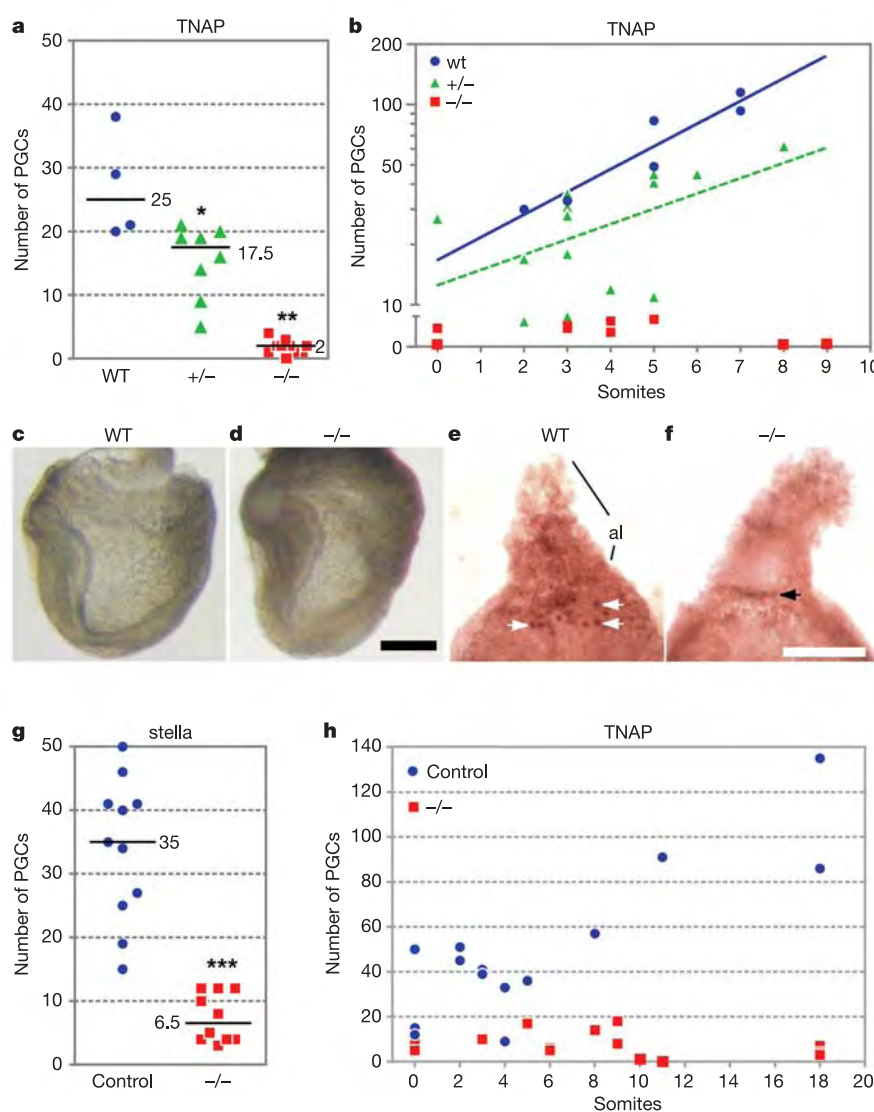


Figure 5 | Loss of PGCs in *Blimp1* deficient embryos. **a**, TNAP-positive founder PGCs at E7.5 (early bud to late head fold stage) in wild-type (WT; $n = 4$), *Blimp1* heterozygote (+/-; $n = 8$) and null (-/-; $n = 10$) embryos. Single asterisk, $P = 0.016$ for WT versus *Blimp1*^{+/-}; double asterisk, $P = 0.002$ for WT versus *Blimp1*^{-/-} embryos. **b**, Linear regression analysis of log PGC number versus somite number at E8.5 for WT (blue solid line, $y = 0.1134x + 1.224$; $n = 6$) and *Blimp1*^{+/-} (green dashed line, $y = 0.0763x + 1.098$; $n = 16$) embryos. **c–f**, Wild-type (**c**) and *Blimp1*^{-/-} (**d**) embryos at E8.5 (2–3 somites, lateral view, anterior to the left) and

corresponding TNAP staining (**e, f**, posterior view) reveal migrating PGCs in wild-type embryos (**e**, white arrows) but only a few clustered cells in *Blimp1*^{-/-} mutant embryos (**f**, black arrow). Al, allantois. Scale bars, 200 μm . **g, h**, PGCs in embryos generated from control and *Blimp1*^{-/-} embryonic stem cells. **g**, stella-positive PGCs in E7.5 control (early bud to late head fold stage; $n = 11$) and *Blimp1*^{-/-} embryos ($n = 10$). Asterisks, $P = 0.0001$. **h**, Comparison of PGCs detected by TNAP staining at E8.5–E9.5 in control embryos ($n = 14$) versus *Blimp1*^{-/-} embryos ($n = 22$). See also Supplementary Fig. 5.

front of the *ROSA26-EGFP* reporter (Fig. 4a, f). The resulting embryos from this cross were collected between the late bud (E7.8) and 3-somite stage, and double-stained with anti-GFP and anti-stella antibodies (Fig. 4b–d). We found that 55–76% of the PGCs (determined by stella expression) were also GFP-positive (Fig. 4e). The reason why not all PGCs were GFP-positive is because of incomplete *Blimp1*-Cre-mediated deletion, which is also reported to occur with other Cre-lines^{26,27}. However, all of the GFP-positive cells (with the exception of one) were also stella-positive (Fig. 4e), suggesting that *Blimp1*-positive cells constitute germline-restricted progenitors of PGCs. Our combined results show that *Blimp1*-positive cells give rise to PGCs, which unequivocally indicates their early lineage restriction.

Mechanism and role of *Blimp1* in PGC specification

To explore whether *Blimp1* is indeed necessary for PGC specification, we created a null allele in which exon 5 of the *Blimp1* gene was flanked by loxP sites and subsequently deleted (see Supplementary Fig. 4). All embryos of different genotypes generated from heterozygous intercrosses were apparently normal until at least E8.5 (Fig. 5c–f and data not shown) and were present in an appropriate mendelian ratio.

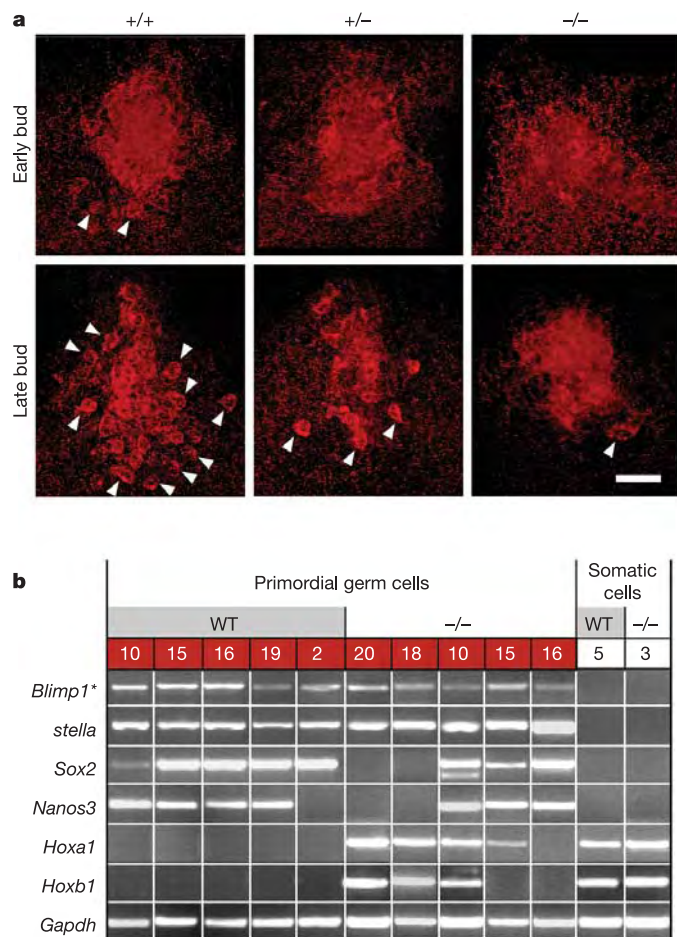


Figure 6 | Aberrant phenotype of *Blimp1* deficient cells. **a**, Posterior views showing fluorescent TNAP staining of *Blimp1* wild-type (+/+), heterozygote (+/-) and homozygote (-/-) embryos. Arrowheads point to PGCs migrating out from the cluster. Scale bar, 50 μ m. **b**, Single-cell cDNA analysis reveals aberrant gene expression in *Blimp1*^{-/-} PGC-like cells at E7.5 (early bud to late bud stage). PGCs were identified by expression of *stella* and *Blimp1* transcripts. The mutant *Blimp1* transcript (denoted by an asterisk) served as a marker only and does not give rise to functional protein (see Supplementary Fig. 4e).

Loss of founder PGCs in *Blimp1* mutants

To examine the fate of PGCs in these *Blimp1* mutant embryos, we stained them for TNAP²³ and counted the clearly identifiable PGCs containing a cytoplasmic spot. At the early bud and early head fold stages of late E7.5 embryos, we detected approximately 25 TNAP-positive PGCs in wild-type embryos. These numbers were reduced to about 17.5 cells in heterozygotes (*Blimp1*^{+/-}), and a maximum of five PGCs were counted in individual *Blimp1* null (*Blimp1*^{-/-}) embryos (Fig. 5a). The relative differences in the number of PGCs in control and mutant embryos are statistically significant, but the total number of PGCs is an underestimate because at these earlier stages, many of the PGCs still form a tight cluster in which individual cells are difficult to count (see below, including Figs 5g and 6a, for further analysis).

At E8.5 (Fig. 5b), the relative numbers of PGCs in homozygous mutants were also very low when compared with control mice. Only a few PGC-like cells were detected near the base of the allantois, and virtually none seemed to be migrating appropriately. Analysis of the numbers of PGCs shows no significant differences in the slopes of the regression lines when comparing wild-type and heterozygous embryos ($P = 0.581$) (ref. 7), suggesting that the dose-dependent effect of *Blimp1* is primarily on the genesis of the founder PGC population and not on their subsequent survival or proliferation. A recent study of *Blimp1* mutant embryos confirms this observation²⁸. Furthermore, we did not detect any abnormalities of the allantois in E8.5 embryos (Fig. 5e, f), suggesting that the *Blimp1*-positive cells are destined for the germ cell fate and are set apart from other epiblast cells that give rise to the allantois and other somatic tissues.

To confirm that the effect of the *Blimp1* mutation on germ cell formation is direct and not a consequence of its expression in the visceral endoderm (which is important for early embryonic patterning²²), we performed 'tetraploid rescue' experiments in which *Blimp1*^{-/-} embryonic stem cells were injected into wild-type host tetraploid blastocysts. The tetraploid host cells contribute almost exclusively to extraembryonic tissues, including the visceral endoderm, while the injected ES cells only contribute to the embryo proper²⁹. We determined the number of PGCs in E7.5 embryos using an anti-stella antibody¹⁰, and detected an average of 35 PGCs in control embryos and 6.5 stella-positive cells in *Blimp1*^{-/-} embryos ($P = 0.0001$) (Fig. 5g). The numbers of TNAP-positive cells at E8.5–E9.5 (Fig. 5h and Supplementary Fig. 5) were also significantly lower in mutants compared with control wild-type mice. The few TNAP-positive PGC-like cells that we detected near the base of the allantois did not seem to be migrating appropriately in the mutants. Taken together, these results demonstrate that the PGC-intrinsic activity of *Blimp1* is essential for the formation of PGCs, and cannot be substituted for by the expression of *Blimp1* within the visceral endoderm.

Aberrant PGC-like cells in *Blimp1* deficient mice

We investigated the phenotypic characteristics of PGC-like cells in mutant embryos at the earlier E7.5 stages (early bud to late bud) using TNAP staining and confocal microscopy (Fig. 6a). At the early bud stage, all the embryos showed a TNAP-positive cluster at the base of the allantois, but only in the wild-type embryos were a few cells migrating out of the cluster. These phenotypic differences were much more prominent at the late bud stage, when most of the TNAP-positive cells containing cytoplasmic 'spots' had started migrating in control embryos. In contrast, the cluster in mutant embryos remained compact and tight, with only a rare migrating cell (Fig. 6a). Furthermore, the number of TNAP-positive cells in control embryos increased from approximately 30 to 58 between the early bud and late bud stages, but their numbers in mutants rarely exceeded 20 at similar stages of development (data not shown); a tight cluster of cells is visible even at E8.5 (see Fig. 5f). It therefore seems that in mutant embryos, the number of cells in the cluster might not increase from the early bud stage onwards, and perhaps

even from the mid-streak stage onwards. Although the number of TNAP-positive cells in heterozygote embryos was similar to those in the null embryos, these cells were more like those in control embryos in that they also seemed to be dispersed and migrating (Fig. 6a). These findings indicate that in the absence of *Blimp1*, TNAP-positive PGC-like cells are formed, but they are aberrant by the early bud stage and they do not proliferate or migrate properly.

***Blimp1* null cells fail to repress *Hox* genes**

Among the TNAP-positive cells in mutant embryos, we detected very few stella-positive cells (Fig. 5g); these cells probably represent the greatest extent to which mutant cells are able to develop any PGC-like characteristics. Using embryos generated from embryonic stem cells in tetraploid hosts, we used single-cell cDNA analysis to compare mutant stella-positive PGC-like cells with cells from control embryos⁹ (Fig. 6b). In control embryos at mid-bud to late bud stages, we identified 7/60 cells that were *Blimp1*- and stella-positive (Fig. 6b). Most of these showed characteristic repression of both *Hoxa1* (5/7) and *Hoxb1* (6/7). However, we also detected four cells that were *Blimp1*-positive and stella-negative, and which showed expression of *Hoxa1* and *Hoxb1* (data not shown). These cells might be at an earlier stage of germ cell development and have yet to induce repression of *Hox* genes, which reinforces the notion that repression of *Hox* genes and expression of *stella* are temporally linked events in PGCs⁹. Almost all somatic cells that were negative for *Blimp1* also showed expression of these *Hox* genes (not shown), which is consistent with the observation that *Hoxb1* expression starts in the mesodermal cells at about the mid-to-late-streak stage³⁰.

From mutant embryos, we detected 5/160 of the relatively rare stella-positive cells expressing the *Blimp1* mutant transcript (see Supplementary Fig. 4e) at the early bud to late bud stage. Notably, 4/5 of these cells showed expression of *Hoxa1* and 3/5 showed *Hoxb1* expression (Fig. 6b), indicating that the repression of *Hox* genes had not occurred consistently in these cells. In contrast, somatic cells from both the control and mutant embryos showed *Hox* expression and were therefore unaffected at least in this respect. Furthermore, we observed that although PGCs from control embryos consistently expressed *Sox2* (7/7) and *Nanos3* (6/7; Fig. 6b and M.S. and M.A.S., unpublished observations), 2/5 mutant cells did not show this expression pattern. Therefore, stella-positive mutant cells seem to be perturbed in several aspects compared to wild-type PGCs. They show inconsistent gene expression patterns, fail to proliferate (Figs 5 and 6a), and may eventually undergo apoptosis or adopt a somatic cell fate.

Discussion

Our study shows that lineage-restricted PGCs are *Blimp1*-positive cells that are first detected in the proximal epiblast and accumulate subsequently. The mechanisms underlying the initiation of *Blimp1* expression in epiblast cells and the accretion of additional *Blimp1*-positive cells from E6.5 onwards are unknown. These cells, which form a cluster of approximately 20 cells at the mid-streak stage, could generate the founder PGCs in a single round of cell division by the early bud stage. In contrast, a previous study on PGC specification using clonal analysis showed that at the E6.5 early streak stage, descendants of single marked cells that contribute to PGCs always include one or two distinct somatic lineages, most predominantly the allantoic mesoderm⁵. Here, we did not detect a contribution of *Blimp1*-positive cells to the allantois or to any other mesodermal cells. It is possible that the clonal analysis in ref. 5 failed to mark one of the few *Blimp1*-positive PGC lineage-restricted cells at E6.5 (Fig. 3a). Assuming this to be the case, after one or more divisions of the marked cells, some of them might subsequently proceed towards the PGC fate after expression of *Blimp1*, while the other descendants contribute to the somatic lineage. Further experiments are needed to trace the fate of the initial *Blimp1*-positive cells before the final PGC specification event.

Our study shows that mutation of the *Blimp1* gene affects the lineage-restricted PGC precursors at a stage that is earlier than the early bud stage, because unlike the cluster in control embryos (where the cells proliferate and subsequently migrate out as PGCs), almost all the cells in mutant embryos fail to proliferate or exit from the cluster. The few stella-positive cells in mutants are also aberrant as they show inconsistent repression of *Hox* genes, which accompanies the final stages of PGC specification in normal embryos. The precise molecular mechanism of *Blimp1* in PGC specification remains to be elucidated.

METHODS

Embryo isolation and single-cell cDNA preparation were performed as described previously^{9,31}. For comparisons between mutant and control embryos (Fig. 6), mutant embryos were generated from *Blimp1*^{-/-} embryonic stem cells, and loxP/loxP embryonic stem cell-derived or normal wild-type embryos were used as controls for the isolation of single cells.

In situ hybridization was performed as described previously³². Antibody stainings on dispersed E7.5 germ cell regions and on whole embryos were carried out essentially as described previously^{25,33}. TNAP staining of PGCs was performed as described previously^{7,34}.

PGC numbers at E7.5 were compared using the nonparametric Mann-Whitney *U*-test and linear regression lines were compared using analysis of covariance (ANCOVA) with GraphPad Prism software.

More detailed methods, concerning particularly the generation of transgenic and knockout mice described in this paper, can be found in the Supplementary Information. The *Blimp1* mutant mice, *Blimp1*-mEGFP mice and *Blimp1*-Cre mice are available from A.T., M.S. and M.N., respectively.

Received 17 March; accepted 10 May 2005.

Published online 5 June 2005.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements We thank S. Chuva de Sousa Lopes, K. Nakao, H. Miyachi and R. Nakayama for technical help, and T. Nakano for anti-stella/PGC7 antibody. B.P. was supported by a Wellcome Trust PhD studentship. M.A.S. is funded by the BBSRC, the Wellcome Trust and the EU Epigenome Programme. M.S. is supported by the Ministry of Education, Culture, Sports, Science and Technology, and a PRESTO grant by the JST. D.O'C. acknowledges the support of the Irvington Institute for Immunological Research and is their National Genetics Foundation Fellow. Thanks to K. Lawson and A. McLaren for discussions and critical comments.

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ARTICLES

Serum response factor regulates a muscle-specific microRNA that targets *Hand2* during cardiogenesis

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Gradients of signalling and transcription factors govern many aspects of embryogenesis, highlighting the need for spatiotemporal control of regulatory protein levels. MicroRNAs are phylogenetically conserved small RNAs that regulate the translation of target messenger RNAs, providing a mechanism for protein dose regulation. Here we show that microRNA-1-1 (*miR-1-1*) and *miR-1-2* are specifically expressed in cardiac and skeletal muscle precursor cells. We found that the *miR-1* genes are direct transcriptional targets of muscle differentiation regulators including serum response factor, MyoD and Mef2. Correspondingly, excess *miR-1* in the developing heart leads to a decreased pool of proliferating ventricular cardiomyocytes. Using a new algorithm for microRNA target identification that incorporates features of RNA structure and target accessibility, we show that *Hand2*, a transcription factor that promotes ventricular cardiomyocyte expansion, is a target of *miR-1*. This work suggests that *miR-1* genes titrate the effects of critical cardiac regulatory proteins to control the balance between differentiation and proliferation during cardiogenesis.

Cellular differentiation and organogenesis involve restricted zones of transcriptional regulation that govern gene expression patterns during specific temporal windows. One mechanism for regulating the target genes activated by transcriptional regulators involves the dose-sensitive response of *cis* elements to gradients of DNA-binding proteins. In this scenario, differences in the levels of transcription factors result in the activation or repression of diverse target genes, allowing finer control of the spatial and temporal events of organogenesis.

MicroRNAs (miRNAs) mediate a recently recognized form of translational inhibition that alters the levels of critical regulators, thereby providing a mechanism for spatiotemporal control of developmental and homeostatic events across a wide range of plants and animals^{1–3}. Genetic studies in *Caenorhabditis elegans* and *Drosophila melanogaster* suggest important functions for specific miRNAs in cell death and proliferation through direct interaction of miRNAs with target sequences in messenger RNAs^{4–11}. However, understanding the specific roles of miRNAs and the regulatory pathways they control has been limited by a lack of reliable and specific methods for identifying miRNA targets.

The transcriptional regulation of cardiomyocyte differentiation and cardiogenesis is highly conserved and requires sequential activation and/or repression of different genetic programmes^{12,13}. Early cardiomyocytes proliferate even as they begin to differentiate, but exit the cell cycle as differentiation progresses. Serum response factor (SRF) binds to CArG boxes in the regulatory region of numerous muscle-specific and growth-regulated genes, and thus has a dual role in regulating the balance between proliferation and differentiation during cardiogenesis^{14–17}. Failure to maintain an adequate pool of undifferentiated myocyte precursors could result in organ hypoplasia, as observed in mice and zebrafish that lack the Hand transcription factor *Hand2* (refs 18–23). Although dynamic temporal and

spatial expression of regulatory pathway components is important in cardiogenesis, whether miRNAs are involved in refining cardiac transcriptional activity is unknown.

Here, we show that the cardiac and skeletal muscle-specific miRNA genes *miR-1-1* and *miR-1-2* are expressed in a chamber-specific manner during cardiogenesis and are activated during the period of differentiation. We find that both genes are direct targets of SRF and its potent co-activator myocardin, and that they are involved in negatively regulating ventricular cardiomyocyte proliferation²⁴. We provide evidence that RNA accessibility is a principal feature of miRNA target recognition, and have incorporated this observation with cross-species sequence matching to identify *Hand2* as an evolutionarily conserved target of *miR-1*. This work reveals a new mechanism for regulating the balance between muscle differentiation and proliferation during organogenesis, and might provide a reliable and specific method for identification of microRNA targets.

miR-1 in developing cardiac and skeletal muscle

To determine whether miRNAs have a role in cardiac development or homeostasis, we searched for miRNAs that were expressed in the mouse cardiovascular system and were conserved across species ranging from flies to humans. On the basis of our *in silico* data and previous reports, the *miR-1* subfamily seemed to be cardiac-enriched^{25,26}. The *miR-1* subfamily consists of two closely related miRNAs encoded by distinct genes that share near complete identity and are designated *miR-1-1* and *miR-1-2* (Fig. 1a). Northern blot analysis revealed that both *miR-1* genes were 21 base pairs (bp) in length and were expressed specifically in the heart and skeletal muscle of adult mice (Fig. 1b and Supplementary Fig. 1).

Owing to the similarity in the *miR-1-1* and *miR-1-2* sequences, and the small size of miRNAs, the relative expression of each *miR-1* gene could not be determined, nor could mRNA *in situ* hybridization

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delineate the embryonic expression domains of the two genes. In order to define the tissue-specific expression and regulation of *miR-1* genes during embryogenesis, we searched for enhancers that might regulate *in vivo* transcription of *miR-1-1* or *miR-1-2*. Comparison of

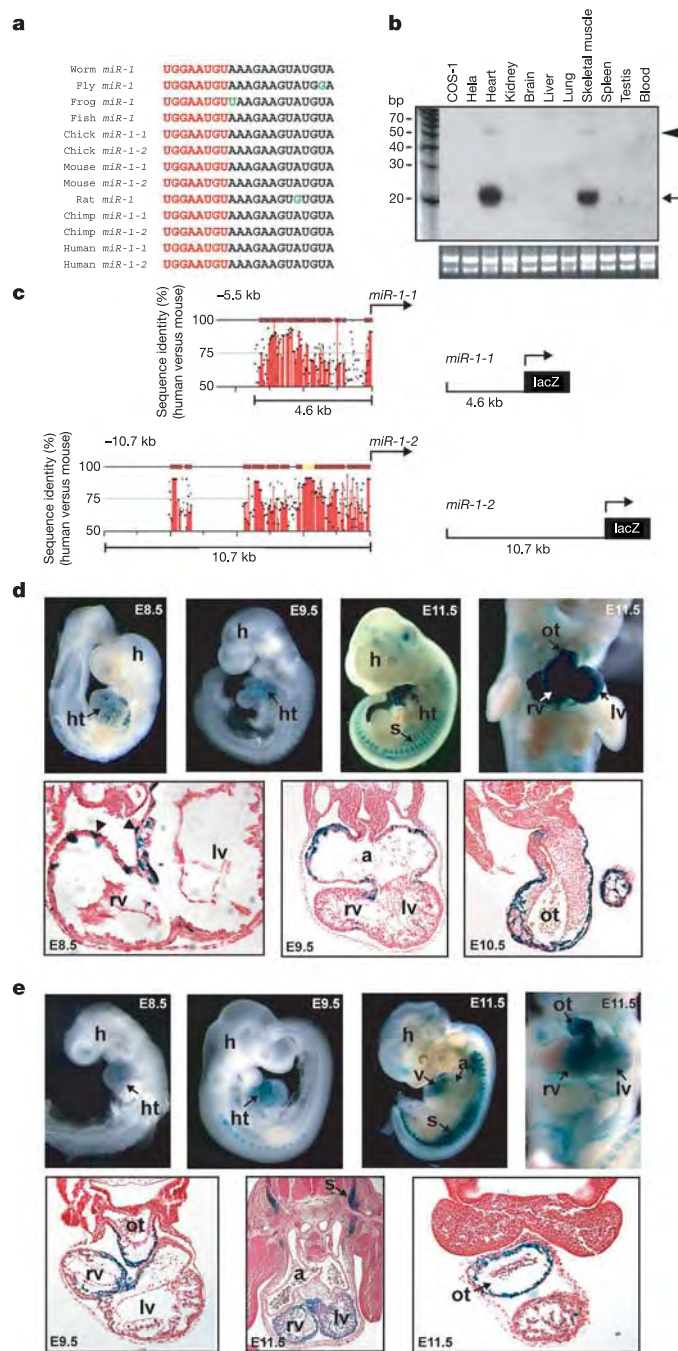


Figure 1 | *miR-1*s are highly conserved and are cardiac- and skeletal-muscle-specific. **a**, Sequence alignment of predicted *miR-1* genes from the indicated species. The eight 5'-nucleotides are highlighted in red; non-conserved residues are in green. **b**, Northern blot of multiple tissues hybridized using a *miR-1*-specific probe. The 70 bp unprocessed form (arrowhead) and 21 bp *miR-1* (arrow) are indicated. **c**, Comparison of the *miR-1-1* and *miR-1-2* promoter regions between mouse and human. Percentage conservation of a 4.6 kb or 10.7 kb genomic region around *miR-1-1* or *miR-1-2* (respectively) is shown. **d, e**, Whole-mounts and sections showing embryonic expression of *miR-1-1* (**d**) or *miR-1-2* (**e**), shown by β -gal activity driven by the genomic fragments indicated in **c**. Arrowheads in **d** indicate the inner curvature. Abbreviations: a, atrium; h, head; ht, heart; lv, left ventricle; ot, outflow tract; rv, right ventricle; s, somites; v, ventricle.

genomic sequences across species using rVISTA revealed that a 4.6 kilobase (kb) and 10.7 kb genomic region around *miR-1-1* and *miR-1-2*, respectively, was conserved between human and mouse (Fig. 1c). The 4.6 kb *miR-1-1* fragment was sufficient to direct lacZ expression in the hearts of transgenic mice after embryonic day (E)8.5 (Fig. 1d), with expression at the early stages being strongest in the inner curvature of the looping heart tube and atria. The inner curvature is less proliferative than the outer curvature, which expands and balloons ventrally to form the cardiac chambers. Cardiac expression of lacZ became more robust and uniform as cardiomyocyte differentiation proceeded, and expression in the myotome of somites also became apparent as skeletal muscle differentiation began (Fig. 1d). Similarly, the 10.7 kb *miR-1-2* fragment contained all the regulatory elements necessary to drive lacZ expression in the embryonic ventricles (but not the atria) and somites at stages corresponding to that described for *miR-1-1* (Fig. 1e). Both *miR-1* enhancers directed expression in the outflow tract of the heart, which arises from a secondary heart field²⁷, distinct from the primary heart field that contributes to atrial and left ventricular myocardium (Fig. 1d, e).

SRF regulates *miR-1* in the heart

Deletion analyses of the *miR-1* enhancers suggested that a 2.6 kb and 0.35 kb region was sufficient for full *miR-1-1* and *miR-1-2* expression, respectively (Fig. 2a, b). Within these regions we noted several *cis* elements conserved between human and mouse that represented potential binding sites for the essential cardiac transcription factors Mef2, SRF, Nkx2.5 and Gata4. SRF sites in the *miR-1-1* and *miR-1-2* enhancers were nearly identical and highly conserved in human, mouse and chick, as were Mef2 sites in *miR-1-1* and MyoD sites in *miR-1-2* (Fig. 2c, d). In transgenic mice, the Mef2 site in the *miR-1-1* enhancer was dispensable for cardiac expression but was necessary for full somite regulation. Mutation of the *miR-1-1* SRF site abolished expression in the heart, and disruption of both the Mef2 and SRF sites abolished all activity of the enhancer (Fig. 2a). Consistent with a cardiac requirement for SRF, mutation of the SRF site in the *miR-1-2* regulatory region disrupted cardiac expression of *miR-1-2*, and mutation of the MyoD site only partially affected somitic expression (Fig. 2b).

In electromobility shift assays, SRF, Mef2 and MyoD could each specifically bind their respective sites in the *miR-1-1* and *miR-1-2* enhancer regions (Fig. 2c, d). SRF is a weak activator of numerous muscle-specific genes and is thought to mediate muscle differentiation by regulating decisions of cellular proliferation and differentiation^{14,15,28,29}. During cardiac and smooth muscle development, the SAP-domain protein myocardin serves as a potent co-activator for SRF and promotes muscle differentiation^{24,30,31}. Consistent with this, we found that SRF was a weak activator of luciferase placed under the control of the *miR-1-1* or *miR-1-2* enhancers, but acted synergistically with myocardin to activate the *miR-1* gene enhancers (Fig. 2e and data not shown); myocardin activity was dependent on an intact SRF-binding site (Fig. 2e). Correspondingly, *miR-1* transcripts were undetectable in RNA from hearts lacking SRF through tissue-specific disruption of the *Srf* gene²⁸ (Fig. 2f). These data suggest that *miR-1-1* and *miR-1-2* function in SRF-myocardin-dependent pathways in cardiac progenitor cells, and are responsive to MyoD/Mef2 in skeletal muscle precursors.

miR-1 regulates ventricular cardiomyocytes

To determine whether the dosage of *miR-1* target genes might be important in the SRF-dependent balance of proliferation and differentiation, we overexpressed *miR-1* specifically in the developing heart under the control of the β -myosin heavy chain (MHC) promoter, which directs high levels of expression by E9.0. Overexpression of *miR-1* resulted in developmental arrest at E13.5, secondary to thin-walled ventricles and heart failure (Fig. 3). The more proliferative compact zone in the *miR-1* transgenic embryos

was only 3–5 cell layers thick, in contrast to non-transgenic littermates, which contained layers of 8–10 cells. Analysis of mitogenic activity using an antibody directed against phosphohistone H3 (Ser 10) revealed a significant decrease in the number of cycling myocardial cells in *miR-1* transgenic mice at E13.5 (Fig. 3), but no increase in apoptotic cells was observed (data not shown). Thus, a decrease in the protein levels of *miR-1* targets during cardiogenesis resulted in a proliferation defect and failure of ventricular cardiomyocyte expansion. Given that *miR-1* expression is regulated by SRF and myocardin, this phenotype is consistent with premature differentiation and early withdrawal of myocytes from the cell cycle¹⁶.

miR-1 targets Hand2 mRNA

Genetic studies in flies and worms have identified several validated *miR-1* targets (Table 1). Recently, several computational methods

have been developed for predicting *miR-1* targets based on the fundamental assumption that the 5'-nucleotides of *miR-1* are most critical for target recognition^{32–37}. Although sequence-based predictions have been successful in plants, the algorithms used to date for non-plant *miR-1* targets often result in few overlapping targets^{34–38}. With few exceptions^{39,40}, large-scale predictions have not yet been validated by demonstration of *miR-1* activity on endogenous targets *in vivo*.

Target accessibility has long been established as an important factor for effective antisense oligonucleotide- and siRNA-mediated silencing⁴¹, and we therefore postulated that it might also be involved in *miR-1* target repression. Using mFold, we analysed all *miR-1* targets identified to date and found that virtually all *miR-1* binding sites in 3'-UTRs were located in 'unstable' regions on the basis of free energy predictions (ΔG) and RNA structure. Table 1 lists the free

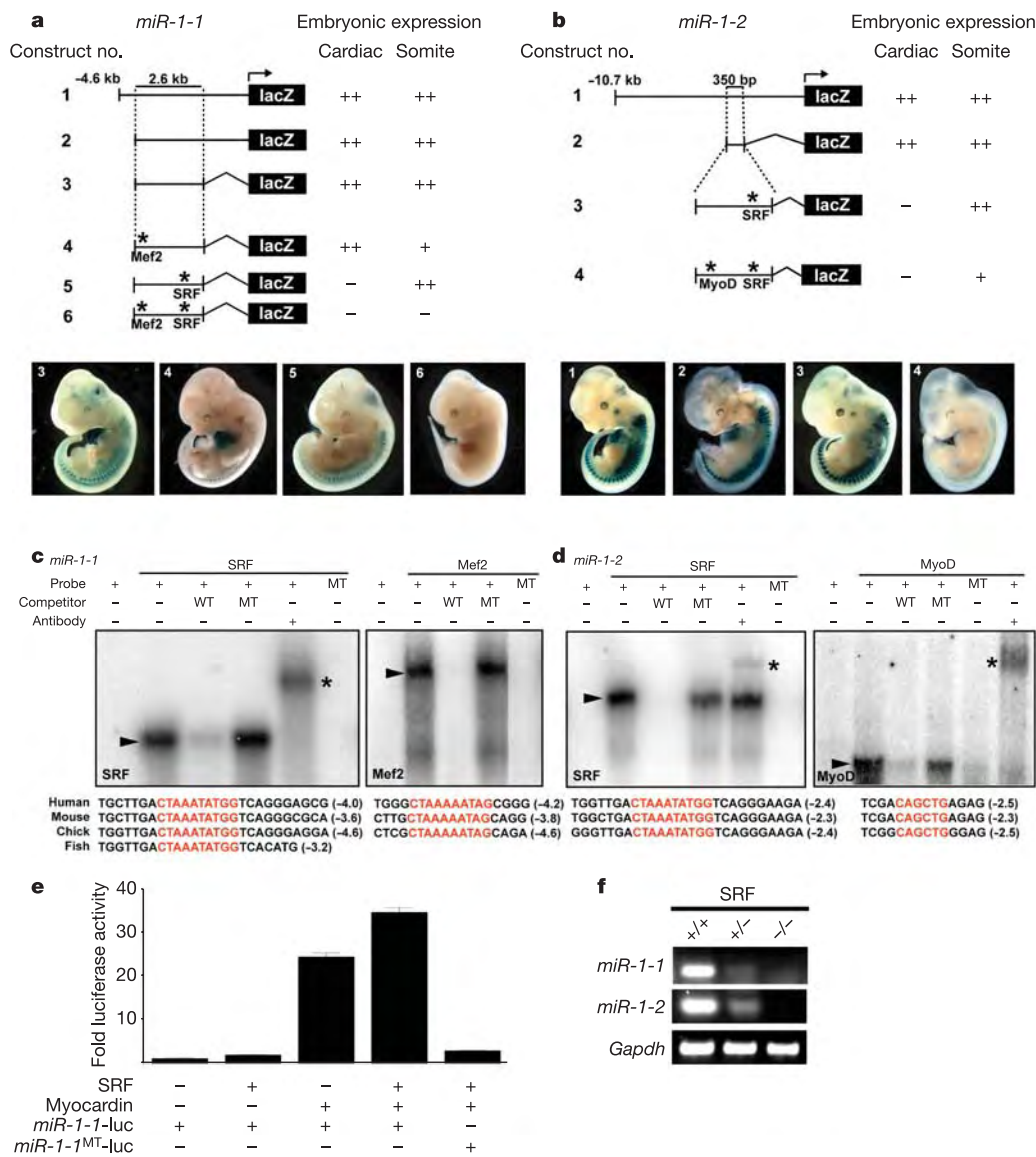


Figure 2 | SRF, Mef2 and MyoD directly regulate embryonic expression of *miR-1*. **a, b**, Deletion and mutation analyses of upstream enhancers of *miR-1-1* (**a**) or *miR-1-2* (**b**). A summary of the effects of mutations (asterisks) on cardiac or somite expression is presented. The bottom panel shows representative images, with construct numbers indicated. **c, d**, Electrophoretic mobility-shift assay using radiolabelled probes (wild type (WT) or mutant (MT)) for SRF, Mef2 or MyoD binding sites (arrowheads). Asterisk indicates supershift in the presence of antibody.

Cross-species conservation of binding sites (with distance upstream of *miR-1*) is shown under the blots. **e**, Fold activation of luciferase downstream of the *miR-1-1* enhancer in COS1 cells by SRF and myocardin with or without a point mutation (MT) in the SRF-binding site. Error bars indicate standard deviations. **f**, PCR with reverse transcription (RT-PCR) of *miR-1* expression in hearts from mice heterozygous or homozygous for cardiac-specific disruption of SRF.

energy of the flanking 70 nucleotides 3' and 5' of the (one or more) predicted miRNA target sequence(s) in the 3'-UTR of validated target genes. The ΔG of the 5' or 3' flanking region around at least one of the predicted miRNA binding sites within the 3'-UTR of each target gene was significantly lower than the average ΔG of 60 random 3'-UTRs (70 bp each) in the corresponding species (Table 1). This suggests a locally linear RNA structure around the target mRNA-binding site that cannot not form tight stems.

However, genes that had multiple putative binding sites typically had several sites that were in regions of high ΔG . To resolve this discrepancy, we compared the conservation of high ΔG versus low ΔG sites in closely related worm ($n = 3$) or fly ($n = 8$) species in an attempt to better predict the validity of true target sites (see Supplementary Fig. 2). We found that virtually all high ΔG sites had sequence differences in the critical 5' region of the miRNA, several of which would clearly disrupt interaction, as seen in *lin-14* (site VI for *lin-4* binding) and *lin-57* (site II for *let-7* binding), making it unlikely that such sites are true targets (see Supplementary Fig. 2). In contrast, every low ΔG site was absolutely conserved across all species, consistent with the idea that they might represent actual target sites (see Supplementary Fig. 2). Consistent with the cross-species data, previous deletion of high ΔG sites in *lin-41* (sites III–VI for *let-7* binding) suggested that they were dispensable. Together, mutational analyses and the conservation of target sites within *Caenorhabditis* and *Drosophila* species support the predictive value of the free energy of sequences flanking true miRNA target sites.

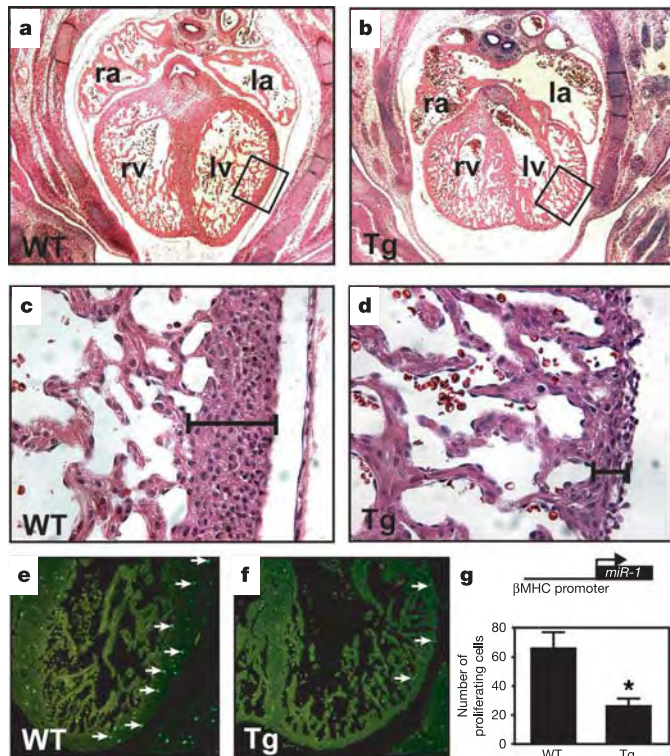


Figure 3 | *miR-1* regulates pool of proliferating ventricular cardiomyocytes and ventricular expansion. **a–d**, Transverse sections of wild-type (**a**) or β -MHC-*miR-1* transgenic (**b**) hearts at E13.5. Boxed areas are shown below at higher magnification (**c**, **d**), with the black bar illustrating the narrowed width of the compact layer in transgenic hearts. **e**, **f**, Immunohistochemical staining in wild-type (**e**) and transgenic (**f**) hearts using an antibody specific to phosphohistone H3, to mark proliferating cells. Arrows indicate proliferating cells. **g**, Quantification of cycling cells shows a statistically significant decrease (asterisk) in the number of proliferating cells in *miR-1* transgenic hearts ($n = 3$). Error bars indicate standard deviations. Abbreviations: la, left atrium; lv, left ventricle; ra, right atrium; rv, right ventricle.

In addition to the flanking sequence, we analysed the stability of the predicted miRNA target sequence itself to determine whether we could find RNA structural features that might affect accessibility and enhance the specificity of target prediction. In a simplified view, the secondary structure of RNA can consist of stems, loops or unstructured single strands. Long stems are stabilizing elements that might render the RNA less accessible to miRNAs. All long loops, including hairpin, interior, bulge and multi-branching loops, could be considered destabilizing elements. Unstructured single strands (including joint sequences and free ends) are also destabilizing, and together with other destabilizing elements, might represent structures that permit miRNA and target sequence interaction. We found that no validated target sites contained stabilizing elements, but most target sequences did have destabilizing elements. Several putative sites that had high ΔG s or had been experimentally dispensable also had stabilizing elements. Consistent with this idea, reported mutations in the spacer region between sites I and II in *lin-41* (ref. 10), which abolished miRNA repression, altered the secondary structure and resulted in the loss of destabilizing elements (Table 1 and Supplementary Fig. 2). There were almost no exceptions to the association of stabilizing elements or destabilizing elements with targets, suggesting that the presence or absence of stabilizing elements and destabilizing elements might have predictive value in an *in silico* screen for putative miRNA targets.

On the basis of the above observations, we searched for putative *miR-1* targets using several assumptions and criteria (Fig. 4a). First, we searched for mRNAs that were complementary to the first eight nucleotides of *miR-1*. Second, consistent with recent reports of free energy of binding⁴², an A to G switch at the eighth nucleotide gave the strongest ΔG , suggesting that a G–U wobble at this position would be allowed (or possibly preferred) for *miR-1* binding to its mRNA targets. Third, we assumed that true 3'-UTR targets would share conservation between chick, mouse, rat and human. Finally, we used mFold to analyse the local mRNA secondary structure 70 bp 5' and 3' of the putative miRNA binding site, selecting for instability within the predicted region, and assessed the secondary structure of the target sequence for the presence of stabilizing or destabilizing elements.

Using these criteria, we scanned all known mRNA 3'-UTRs as potential *miR-1* targets. Approximately 13 mRNA 3'-UTRs matched *miR-1*, and the putative target sequences were conserved across species (Fig. 4b). However, most 5' and 3' flanking regions had ΔG s that were close to the species average, and did not suggest local instability (Fig. 4b). To validate putative targets and to test whether the ΔG and stabilizing/destabilizing elements would add further specificity to our *in silico* screen, we selected for further study a few predicted targets that were co-expressed with *miR-1* genes in the heart or skeletal muscle. One of these, the transcription factor *Hand2* had a particularly unstable 5' region, with a ΔG of $-4.6 \text{ kcal mol}^{-1}$. Animals lacking *Hand2* show characteristic failure of ventricular cardiomyocyte expansion^{18,20,22,23}. We also tested thymosin $\beta 4$, a cardioprotective G-actin-sequestering protein expressed during cardiogenesis⁴³ that does not have a predicted ΔG lower than the average but shows high sequence complementarity with *miR-1* (see Supplementary Fig. 3).

Previous studies suggest that miRNA-binding sites are transferable and sufficient for conferring miRNA-dependent translational repression^{39,42}. To test this, we placed multimers of about 80 bp containing the predicted *miR-1* target site from *Hand2* or thymosin $\beta 4$ 3'-UTRs into the 3'-UTR of a luciferase reporter plasmid (Fig. 4c). We introduced the luciferase expression vector under a constitutively active promoter with or without *miR-1* into COS1 cells and measured the level of luciferase enzyme activity to determine the effects of *miR-1* on luciferase translation. Transfection of the *Hand2* chimaeric luciferase reporter into COS1 cells, which do not express any endogenous *miR-1* (Fig. 1b), consistently resulted in decreased luciferase activity when wild-type *miR-1* was also transfected

Table 1 | *In silico* analysis of previously described miRNA targets

Organism	miRNA	Target	Site	ΔG for 5' 70 bp ($-kcal\ mol^{-1}$)	ΔG for 3' 70 bp ($-kcal\ mol^{-1}$)	DSE	SE
<i>C. elegans</i> (avg. $\Delta G = 7.2$)	Lin-4	<i>lin-14</i>	I	7.7	3.4	-	-
			II	7.8	1.5	IL	-
			III	2.6	4.2	HL	-
			IV	5.0	8.3	-	-
			V	4.2	10.6	-	-
			VI	9.3	8.7	-	Stem
			VII	3.2	3.0	Joint	-
		<i>Lin-28</i>	I	0.6	10.1	Free end	-
		<i>lin-57</i>	I	10.2	8.2	Joint	-
	let-7	<i>lin-41</i>	II	3.1	4.8	HL	-
			I	0.6	7.6	Joint	-
			II	9.4	7.2	MBL	-
			III	6.2	7.2	IL	-
			IV	8.1	6.3	IL	-
			V	5.1	9.4	HL, free end	Stem
			VI	12.4	5.9	HL	Stem
		<i>lin-41</i> (MT)	I	0.6	4.4	-	-
			II	6.6	7.2	-	-
		<i>daf-12</i>	I	1.6	10.2	IL	-
			II	8.9	1.1	HL, free end	-
		<i>lin-57</i>	I	1.5	3.9	MBL	-
			II	8.1	8.8	-	-
			III	5.9	4.7	Free end	-
			IV	2.3	0.7	MBL	-
			V	0.3	0.1	HL	-
			VI	0.2	0.3	HL, free end	-
			VII	0.7	3.8	Free end	-
			VIII	0.3	9.0	Free end	-
	lsy-6 miR-273	<i>cog-1</i>	I	1.1	1.7	Free end	-
		<i>die-1</i>	I	3.8	0.3	Free end	-
			II	4.1	5.5	MBL	-
<i>D. melanogaster</i> (avg. $\Delta G = 8.5$)	bantam	Hid	I	3.4	8.6	HL	-
			II	9.5	9.2	HL	-
			III	1.3	4.1	Free end	-
			IV	24.7	19.2	-	-
			V	8.6	7.2	Joint	-
<i>M. musculus</i> (avg. $\Delta G = 13.4$)	miR-196	<i>Hoxb8</i>	I	12.8	1.6	HL	-
	miR-375	myotrophin	I	9.2	7.2	-	-

Free energy (ΔG) analysis of sequence flanking each putative target binding site, and description of the destabilizing elements (DSE) or stabilizing elements (SE) within binding sites. HL, hairpin loop; IL, interior loop; MBL, multi-branching loop; MT, mutant.

(Fig. 4d). This was uniformly true for the putative miR-1 target region from the 3'-UTR of mouse, chick, frog or fish, suggesting evolutionary conservation of *Hand2* as a target of miR-1 (Fig. 4d and Supplementary Fig. 4). In this assay, the 3'-UTR of thymosin $\beta 4$ also resulted in decreased luciferase activity. A mutant target sequence of *Hand2* or thymosin $\beta 4$ fused to the 3'-UTR of luciferase was not responsive to wild-type miR-1, suggesting specificity of the repression effect. Furthermore, mutant *miR-1* genes (see Supplementary Fig. 4) had no effect on the wild-type target sequences, but could partially repress translation of luciferase transcripts containing the complementary mutant 3'-UTRs (Fig. 4d and Supplementary Fig. 4).

Although the ability of miRNAs to repress translation of chimaeric luciferase reporters is a useful screening tool, it remains a surrogate for testing the effects of miRNAs on their putative targets and can result in misleading assessment of targets. To more directly test the validity of putative targets, we asked whether miR-1 could repress endogenous protein expression *in vivo* in transgenic mice (Supplementary Fig. 5). Western blots of transgenic heart protein overexpressing *miR-1* postnatally showed a significant decrease in *Hand2* protein levels compared with non-transgenic littermates, confirming *Hand2* as a miR-1 target *in vivo* (Fig. 4e). No change in mRNA levels of *Hand2* was noted (Fig. 4e). Together, the *in silico*, *in vitro* and *in vivo* data provide compelling evidence that *Hand2* is a true target of miR-1 during cardiogenesis. In contrast, we did not detect any difference in thymosin $\beta 4$ protein levels in the *miR-1*-overexpressing transgenic hearts compared with wild type, despite perfect sequence complementarity and conservation (Fig. 4e and Supplementary Fig. 3). This is consistent with the regions of high ΔG around the

target sequence of thymosin $\beta 4$. Similarly, despite the low ΔG for another putative target, insulin-like growth factor 1 (*Igf1*), we did not detect any change in IGF1 protein levels in transgenic hearts, consistent with the presence of a stabilizing element in the target sequence (Fig. 4b).

Discussion

Here we have described a conserved cardiac- and skeletal-muscle-specific regulatory pathway involving an SRF-myocardin-dependent miRNA that regulates translation of the central cardiac transcription factor *Hand2*. The early exit of cardiomyocytes from the cell cycle upon overexpression of *miR-1* might reflect the role of miR-1 downstream of SRF and myocardin, to more finely regulate the balance between cell proliferation and differentiation. The potential role for miR-1 s in mediating the effect of MyoD on skeletal muscle differentiation might be equally important and awaits future studies.

The observation that *miR-1* expression begins after cardiac looping and becomes robust only later is consistent with a model in which temporal regulation of *Hand2* activity is necessary for cardiac differentiation. Through regulation of *Hand2* (and probably other critical factors), miR-1 s appear to regulate their targets temporally and spatially, contributing to multiple aspects of cardiogenesis. This type of regulation might be common during embryogenesis in order to titrate the effects of critical signalling and transcriptional pathways, and allow appropriate decisions regarding cell fate, proliferation and differentiation.

The algorithm we used to predict miRNA targets is based on observations from previously validated targets and is an attempt to

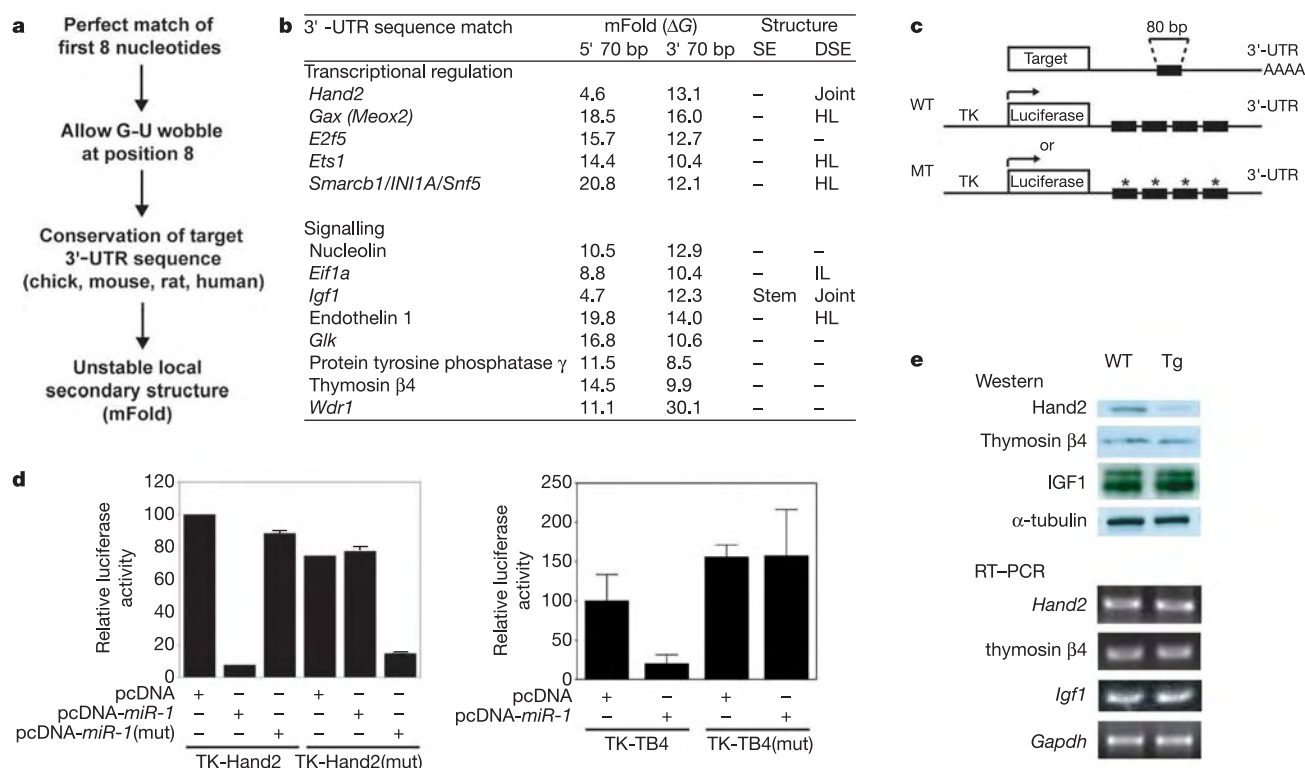


Figure 4 | Prediction and validation of miR-1 targets. **a**, Algorithm for *in silico* prediction of microRNA targets. **b**, List of mRNA sequences with conserved sequence matching with *miR-1* across species. Predicted ΔG ($-\text{kcal mol}^{-1}$) of 70 bp 5' and 3' flanking regions neighbouring potential target sites is shown. Presence of stabilizing elements (SE) or destabilizing elements (DSE) in the target sequence structure. **c**, Approach for testing the transferability of the *miR-1* target sequence to the luciferase reporter using

multimerized copies of wild-type or mutant (asterisks) sequence. TK, thymidine kinase promoter. **d**, Relative luciferase activity under various conditions using the 3'-UTR target sequence of mouse *Hand2* and the target sequence of thymosin β 4 transferred to the luciferase 3'-UTR (TK-Hand2 or TK-TB4, respectively). Error bars indicate standard deviations. **e**, Western blot of protein from wild-type hearts and transgenic hearts overexpressing *miR-1*. RNA transcript levels were equal, as seen by RT-PCR (bottom panel).

develop certain 'principles' that appear to be followed by known miRNAs and their targets^{4–11,39,40,44}. The major difference between our approach and that of others is the additional evaluation of the energy states of sequences flanking the miRNA target (ΔG) and the presence or absence of stabilizing/destabilizing elements in target RNA. Consistent with RNA secondary structure having an important role in miRNA target accessibility, ATP-assisted unwinding of RNA secondary structure does not seem to be involved in target recognition by short interfering (si)RNA^{45,46}. We propose a model in which miRNAs preferentially target 3'-UTR regions with less complex secondary structure that are more accessible. The criteria described here may lead to increased specificity of target prediction, perhaps at the expense of sensitivity. Formal testing of this model will be possible as additional, biologically validated microRNA targets are identified.

METHODS

Bioinformatics. Multiple sequence alignment was performed with ClustalX 1.83 using appropriate settings, and promoter analysis was performed with rVISTA. PatScan⁴⁷ program was used for target searches, and energy states and RNA folding were determined using mFold⁴⁸. Average ΔG values in each species were determined by randomly selecting 60 3'-UTR fragments of 70 bp in length.

We determined the RNA secondary structure of each miRNA-binding site plus 30 bp flanking sequence on each side. On the basis of our observations, we set the following values as a cutoff to define stabilizing and destabilizing elements. Average stem length was calculated for each species from at least 60 randomly selected sequences, and cutoff for a stabilizing element stem was defined as: ≥ 8 bp (worms), ≥ 9 bp (flies), ≥ 10 bp (mice). Loops or unstructured single strands were defined as destabilizing elements with the following cutoff lengths: hairpin loop, ≥ 11 bp; interior loop, ≥ 9 bp; bulge loop, ≥ 7 bp; multiple-branching loop, ≥ 11 bp; joint sequence and free end, ≥ 11 bp.

Plasmid construction. Target sequences and their mutant forms were synthesized as DNA oligonucleotides. After annealing and concatamerization, four copies of the target sequence were excised using a sized gel, blunt ended and sub-cloned into a pGL-TK vector. To express *miR-1* genes in COS1 cells, the genomic sequence containing pre-*miR-1* gene sequences plus about 50 bp flanking each side were inserted into pcDNA3. Site-directed mutagenesis by polymerase chain reaction (PCR) was performed using Pfu DNA polymerase.

Cell transfection, EMSA, luciferase assay, northern and western blotting. Plasmid transfection was performed in 12-well plates using FuGENE 6 (Roche). An RSV-lacZ expression construct was co-transfected to normalize for transfection efficiency. Luciferase and β -galactosidase activities in the cell extract were assayed 36 h after transfection using a luciferase assay system (Promega). All experiments were repeated at least three times, and representative results are shown. Northern blotting was performed as previously described²⁵. Western blotting was performed on heart lysates using standard methods with specific antibodies. Electrophoretic mobility-shift assays (EMSA) were performed as previously described⁴⁹.

Transgenic mice. Transgenic mice were generated and β -galactosidase staining and histological analyses were performed as previously described⁴⁹. For promoter analysis, different fragments were subcloned into a pHsp68lacZ reporter vector. For overexpression studies, pre-*miR-1* plus flanking sequence was subcloned into α -MHCclone26 or β -MHCclone32 vectors.

Received 7 March; accepted 17 May 2005.

Published online 12 June 2005.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements We wish to thank K. Ivey for critical discussions and for preparation of figures; members of the Srivastava laboratory for helpful discussions; J. McAnally for generation of transgenic mice; E. N. Olson for plasmids; and R. Misra and R. Balza for providing SRF-null embryonic heart cDNA. D. S. was supported by grants from NHLB/NIH, the March of Dimes Birth Defects Foundation and the American Heart Association.

Author Information MicroRNA 1 sequences have been deposited in Genbank under the following accession numbers: DQ066648 and DQ 066649 (*Pan troglodytes* pre-microRNA-1-1 and 1-2), DQ066650 (rat pre-microRNA-1), DQ066651 (*Danio rerio* pre-microRNA-1) and DQ066652 (*Xenopus tropicalis* pre-microRNA-1). Reprints and permissions information is available at npg.nature.com/reprintsandpermissions. The authors declare no competing financial interests. Correspondence and requests for materials should be addressed to D.S. (dsrivastava@gladstone.ucsf.edu).

Mammalian mutagenesis using a highly mobile somatic *Sleeping Beauty* transposon system

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Transposons have provided important genetic tools for functional genomic screens in lower eukaryotes but have proven less useful in higher eukaryotes because of their low transposition frequency. Here we show that *Sleeping Beauty* (SB), a member of the Tc1/mariner class of transposons, can be mobilized in mouse somatic cells at frequencies high enough to induce embryonic death and cancer in wild-type mice. Tumours are aggressive, with some animals developing two or even three different types of cancer within a few months of birth. The tumours result from SB insertional mutagenesis of cancer genes, thus facilitating the identification of genes and pathways that induce disease. SB transposition can easily be controlled to mutagenize any target tissue and can therefore, in principle, be used to induce many of the cancers affecting humans, including those for which little is known about the aetiology. The uses of SB are also not restricted to the mouse and could potentially be used for forward genetic screens in any higher eukaryote in which transgenesis is possible.

Transposon-tagged mutagenesis has proven invaluable for functional genomic screens in organisms such as *Drosophila melanogaster*^{1,2}, *Caenorhabditis elegans*³ and plants⁴, but the lack of active elements in higher eukaryotes has precluded their use for mammalian functional genomics. This problem was partly solved when molecular reconstruction was used to produce a transposon, *Sleeping Beauty* (SB), that transposes in higher eukaryotic cells⁵. There is much interest in these transposons as insertional mutagens because they have a small target site for integration (for SB, this is TA) and require few host cell factors for their activity. SB is a bipartite transposition system consisting of the transposase and transposon vector, which is flanked by the binding sites for the SB transposase, the so-called inverted repeats/direct repeats (IRDRL (left) and IRDRR (right)). Transposons are mobilized by SB transposase supplied *in trans*. SB is active in the mouse germ line^{6–8} (one or two transpositions per animal born) and mouse somatic cells^{7,9} but the transposition frequency is too low to be useful for most genetic screens¹⁰.

Analysis of SB transposition integration sites cloned from the mouse germ line indicates that SB has fewer transposition site biases than retrotransposons, increasing its potential as an insertional mutagen^{9,11}. However, SB does show a small but significant bias towards genes and their upstream regulatory sequences, although this bias is much less than that observed with retroviruses¹². SB elements are also not locked in place after transposition and can continuously transpose to new sites. These qualities would make SB an attractive insertional mutagen for functional genomic screens in higher eukaryotes if the frequency of transposition could be increased.

A limitation of SB is that transposed elements tend to reintegrate at sites linked to the donor site. Previous studies showed that 50–80% of germline SB transpositions are located within 10–25 megabases of the donor site^{7–9}. This problem can also be overcome

by increasing SB transposition frequencies to levels in which local transposition is no longer rate limiting for genome-wide insertional mutagenesis.

Creating a highly active SB mutagenesis system

To develop a more active eukaryotic SB transposition system we made several enhancements to the SB transposition system used previously. We generated a mutagenic transposon vector, T2/Onc2 (Fig. 1a). This transposon is similar to that described by an accompanying paper (ref. 13) but contains a larger fragment of the *engrailed-2* (*En2*) splice acceptor (SA) and is flanked by optimized SB transposase binding sites that increase SB transposition¹⁴. It is also smaller than other SB transposons used previously (about 2.0 kilobases (kb)) and approaches optimal size for transposition¹⁵. T2/Onc2 contains two splice acceptors and a bi-directional poly(A) and can terminate transcription when integrated in either orientation in a gene. It also contains a murine stem cell virus (MSCV) long terminal repeat (LTR) and a splice donor (SD) and can promote gene expression when integrated upstream or within a gene. Thirty T2/Onc2 transgenic founders were generated after microinjection. Because SB transposes by a cut-and-paste mechanism, the number of transposons in the transgene concatamer can initially limit the number of transposition events. Any methylation present on the transposon could also be transferred to new sites within the genome. Methylation of the MSCV promoter might therefore inhibit its ability to affect expression of neighbouring genes. With this in mind, founder transgenic animals were screened to determine their transposon copy number and the methylation status of the MSCV promoter (Fig. 1b, Supplementary Fig. 1). Three founder transgenic animals containing a high copy number of unmethylated transposons (6057, 6070, 6113) were used to establish transgenic lines (Fig. 1b). Transposon concatamers from each line were transmitted

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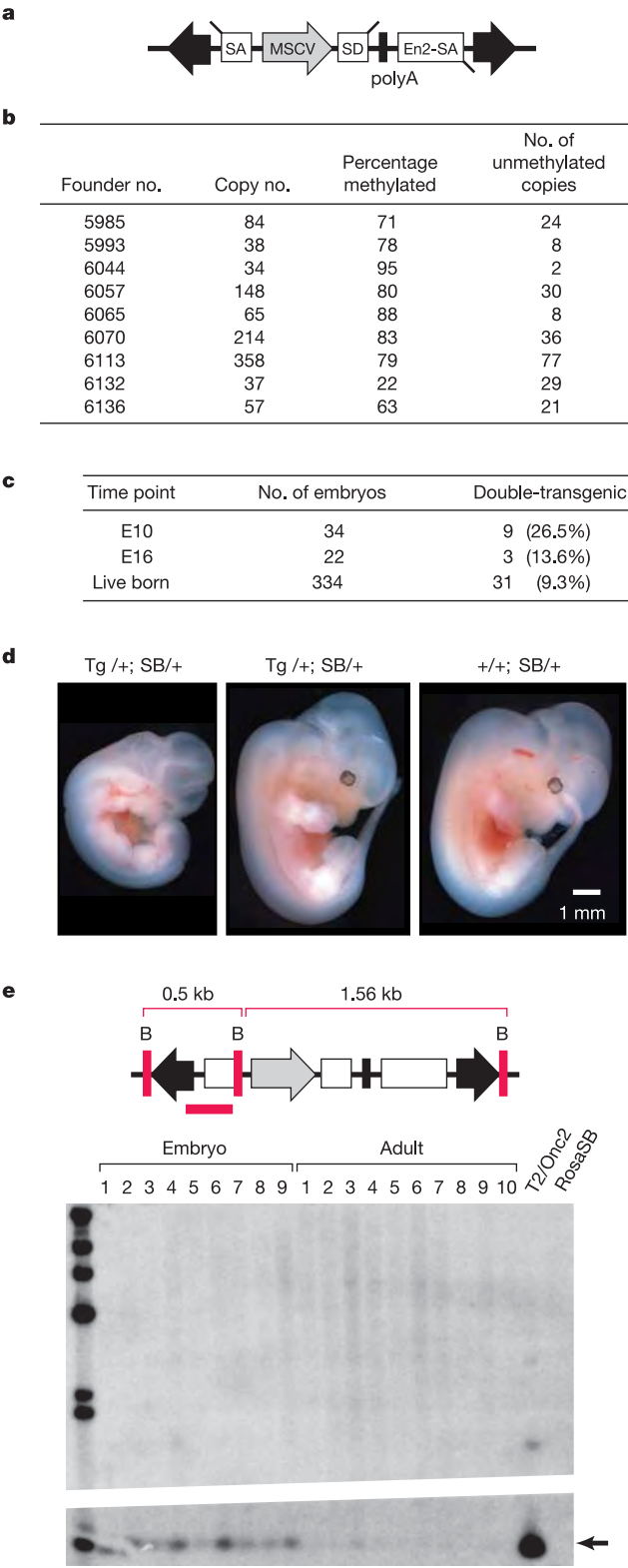


Figure 1 | Analysis of double-transgenic embryos and adults. a, Structure of the T2/Onc2 transposon. **b**, Estimates of transgenic transposon copy number, and percentage of methylated transposons determined after digestion with *DraI*-*MspI* or *DraI*-*HpaII*. **c**, Reduced number of E16 double-transgenic embryos and adults. **d**, Double-transgenic embryos (left panel) were often smaller than control littermates. **e**, The 500-bp *Bam*HI concatemer fragment (arrow) is reduced in intensity in double-transgenic embryos and adults relative to the T2/Onc2 heterozygous transgenic control. For adult tissues, brain DNA is in the odd-numbered lanes and kidney DNA is in the even-numbered lanes.

at normal mendelian frequencies, and heterozygous mice showed no obvious phenotype.

Next, we generated a transposase knock-in allele to avoid the epigenetic silencing often seen with transgenes. To increase SB transposition we generated the knock-in by using the SB11 transposase¹⁵. This transposase contains four amino acid substitutions that increase its activity above that of the SB10 transposase used previously. An expression cassette consisting of a splice acceptor site upstream of the SB11 complementary DNA followed by an SV40 polyadenylation signal was targeted to the *Rosa26* locus to generate the *RosaSB* allele (Supplementary Fig. 2). This site was chosen because genes targeted to this locus are expressed ubiquitously during development and in adult mouse tissues. Western blotting confirmed the expression of the SB11 transposase in *RosaSB* mice (data not shown), and quantitative polymerase chain reaction (PCR) indicated that the *RosaSB* allele is equally expressed in all tissues tested (brain, spleen, skin and lung) (data not shown). Heterozygous *RosaSB* mice were aged for more than a year and showed no obvious phenotype.

RosaSB mice were then crossed to each T2/Onc2 transgenic line to generate a cohort of mice harbouring both elements. Unexpectedly, intercross offspring showed a non-mendelian inheritance pattern with a significant decrease in progeny inheriting both the *RosaSB* transposase and the T2/Onc2 transgene. All three T2/Onc2 lines produced fewer double-transgenic progeny than expected, although the frequency varied between the lines (TG6070, 17/136 (12.5%); TG6057, 5/89 (5.6%); TG6113, 9/109 (8.3%)). We proposed that this decrease in viability was due to lethality induced by SB transposition and/or DNA damage that was not repaired after SB excision. Previous studies with mice deficient for proteins involved in nonhomologous end joining indicate that lymphocytes and neurons are particularly sensitive to double-strand breaks during development^{16,17}. To test this hypothesis, we characterized embryos at various developmental time points. Normal frequencies of double-transgenic embryos were observed at embryonic day 10 (E10), whereas a significant decrease was seen by E16 (Fig. 1c). Embryos at both time points seemed grossly normal, although many double-transgenic embryos seemed smaller than control littermates (Fig. 1d). Histopathological examination of double-transgenic embryos showed various developmental abnormalities unique to each embryo (data not shown).

To determine whether SB transposition occurs in double-transgenic embryos, we asked whether SB transposons had been excised from transposon concatomers in double-transgenic embryos, because excision is the first step in transposition. *Bam*HI sites are located within the plasmid sequences that flank each transposon in the concatomer and in the transposon itself (Fig. 1e). Consequently, any transposon in the concatomer will generate a 500-bp fragment by using the probe indicated. It is unlikely that *Bam*HI sites will immediately flank a transposon after transposition. Reintegrated transposons will therefore primarily generate *Bam*HI fragments that are larger than 500 bp. Analysis of nine double-transgenic embryos showed that most transposons were excised from the concatomer by E10 (Fig. 1e). Analysis of the brain and kidney of ten adult double-transgenic animals showed that transposon excision continues in the adult, until by postnatal day 45 virtually all of the transposons within the concatomer have been excised (Fig. 1e). Excision therefore begins early in development and continues into the adult, affecting virtually all cell types.

Previous studies showed that 75% of excised transposons re-integrate into the mouse genome¹⁰. To confirm that excised transposons re-integrate into the mouse genome we used ligation-mediated PCR (LM-PCR)¹⁸ to amplify SB junctions from ten double-transgenic embryos. LM-PCR is a powerful new amplification method that makes it possible to amplify and sequence thousands of SB transposition sites rapidly (Supplementary Methods). Ninety-six SB junction fragments were picked randomly and sequenced from each amplified embryo library (Supplementary Table 1). BLAST

searches of the 490 independent transposon junctions showed that most junctions were rare and represented only once among the clones analysed (Supplementary Table 1), indicating that each junction is present in a limited number of cells. This is consistent with Southern blot data, which showed no detectable newly acquired SB transposons in double-transgenic embryos (Fig. 1e). SB transposons are therefore reintegrating into the mouse genome at many sites in double-transgenic mice.

T2/Onc2 concatemer integration sites are located on chromosomes 1, 4 and 6 (data not shown). As expected, we saw an increased frequency of transposons reintegrated on these chromosomes in double-transgenic embryos. The percentage of local transposition within a 25-megabase region varied from 6% to 11% in double-transgenic embryos (data not shown) in contrast to germline transpositions reported by others, in which 50–80% of the transpositions were local^{7–9}. Even when transposons landed on the same chromosome as the transgene concatemer, the transposon integrations were well distributed across the chromosome and there was no easily defined local hopping interval. We speculate that the higher SB transposition frequencies obtained with our system permit secondary and tertiary rounds of transposition, which masks local transposition. This high rate could be attributed to a more optimal expression of the SB11 transposase from the RosaSB allele. Previous work has indicated that even moderate changes in SB transposase expression can have a significant impact on transposition frequency¹⁵.

SB transpositions in the embryo are fairly well distributed across the genome. When T2/Onc2 integrated in or near a gene there was little preference for a gene region or orientation relative to the nearest gene (Supplementary Tables 1 and 5). Only four regions in the

genome (less than 30 kb in size) contained two SB transposon integrations in independent embryos (Supplementary Table 2), which is similar to the number (3) predicted by Monte Carlo simulations for random integration, and no region (less than 100 kb in size) contained three SB transposon integrations. The embryo data therefore seem to represent a population of unselected transposon integrations.

Double-transgenic mice are tumour-prone

Twenty-four double-transgenic mice that survived to weaning were monitored for tumour development. At 7 weeks of age the mice began to show signs of illness, and by 17 weeks all the mice had died from cancer (Fig. 2a). Multiple tumour types were identified: the most common tumour was T-cell lymphoma (Fig. 2b). Tumour cells were frequently found in all tissues of the animal and in some cases a single animal developed two or even three different cancer types (Fig. 2b). Haematopoietic tumours predominated, possibly reflecting the large pool of haematopoietic stem cells present in mice. Medulloblastoma, a solid tumour of the cerebellum, was also observed in two mice; intestinal and pituitary neoplasia was seen in other animals. Thus, unlike retroviral insertional mutagenesis, SB mutagenesis is not limited to the haematopoietic system. Stained sections of the medulloblastoma from animal TG6057-17106 (Fig. 3a, c) and a corresponding normal cerebellum (Fig. 3b, d) showed that the normal morphology of the cerebellum was disrupted, with tumour cells invading the molecular layer (Fig. 3a). Tumour tissue also extended down the brain stem and could be seen adjacent to the spinal cord (Fig. 3c). This is similar to what is observed in human medulloblastoma.

BamHI-digested tumour DNAs were subsequently analysed by

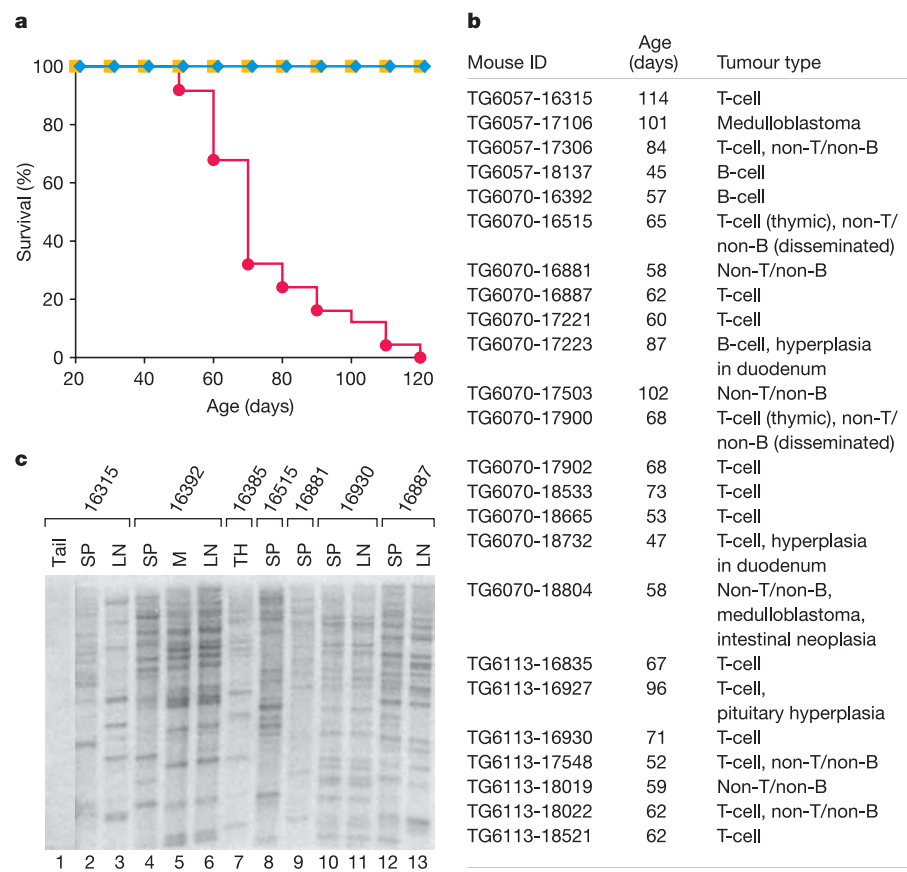


Figure 2 | Adult double-transgenic mice die from cancer. **a**, Survival curves showing decreased viability of double-transgenic mice; yellow, RosaSB; blue, T2/Onc2; red, double-transgenic. **b**, Age at death and tumour type of

double-transgenic mice. **c**, Southern analysis of BamHI-digested tumour DNA. Each band represents a separate SB transposon integration. LN, lymph node; M, mass; SP, spleen; TH, thymus.

Southern blotting to determine whether they contained clonal or subclonal SB transpositions. As expected, Southern blotting failed to identify clonal, somatically acquired transposon integrations in tail DNA (Fig. 2c, lane 1) or in DNA from normal brain and kidney (Fig. 1e). In contrast, numerous clonal and subclonal transposon integrations were seen in lymph nodes, spleen and thymus in tumour DNA (Fig. 2c, lanes 2–13). The pattern of transposon integrations in different tumour tissues from the same animal was similar but not identical (Fig. 2c, lanes 4–6, 12 and 13), indicating that some transpositions are lost while others are gained during tumour development. These results are consistent with insertional mutagenesis of cancer genes as the disease-inducing mechanism.

Analysis of SB integration sites in tumour DNA

To confirm that these tumours are induced by insertional mutagenesis, we cloned and analysed 781 SB junctions from 16 tumours (Supplementary Table 3). In contrast to the results from embryos (Supplementary Table 2), we identified multiple genes that were mutated by SB integration in two or more tumours (Supplementary Table 4). These results are unlikely to have occurred by chance. Seven of these genes are validated human cancer genes, and a further seven are mutated by retroviral integration in mouse leukaemias (<http://rtcgd.ncifcrf.gov>). We also identified four genes that were mutated more than once in the same tumour (two *Notch1* integrations (TG6057-16315), two *Jak1* integrations (TG6070-16887), two *Csf3r* integrations (TG6070-17306), two *Erg* integrations (TG6070-17900) and three *Erg* integrations (TG6070-16881)) (Supplementary Table 4). This could reflect tumour microheterogeneity, with the different integrations occurring in different subpopulations of tumour cells during tumour progression. We also identified integrations in several genes that have not yet been examined for a role in human cancer but which represent excellent disease gene candidates (Supplementary Table 4).

Like the embryo integrations, tumour integrations were widely distributed across the genome with little local hopping (data not shown). However, integrations in tumour DNA located upstream or within genes showed an orientation bias that was not found in

embryo integrations (Supplementary Table 5). In tumours, 65% of transposons located 5' of genes are in the same transcriptional orientation as the gene, compared with 52% for integrations in embryos ($P < 0.001$). In addition, 62% of transposons located within genes are in the same orientation, compared with 54% for integrations in embryos ($P < 0.001$). Unlike retroviruses, which have strong enhancer activity and can activate gene expression over large distances, T2/Onc2 seems to have little enhancer activity. This is supported by our failure to identify common integration sites in which transposons are integrated downstream of the gene (Supplementary Table 4) and by recent data showing that SB transposons that lack viral LTR and corresponding SD sequences fail to increase the expression of nearby genes significantly¹². Consequently, T2/Onc2 primarily activates gene expression by integrating upstream of a gene or in an upstream intron and promoting the expression of the gene from the MSCV LTR, or by integrating into the coding region and either promoting the expression of a truncated protein or truncating the transcript prematurely (Supplementary Table 4; data not shown). This lack of enhancer activity greatly simplifies the identification of cancer genes mutated by SB.

Activating *Notch1* transpositions

Activating *NOTCH1* mutations have been identified in more than 50% of human T-cell acute lymphoblastic leukaemia (T-ALL) cell lines¹⁹. Among ten SB-induced T-cell lymphomas analysed, six contained SB integrations in intron 27 of *Notch1* (Fig. 4a, Supplementary Table 4). These transposon integrations mapped to three different sites in intron 27, indicating that transposition is not totally random or that integration at these sites is selected because of their effect on *Notch1* expression. All six integrations are oriented in the same transcriptional direction as *Notch1* and induce the expression of a *Notch1* fusion transcript containing the MSCV promoter and the

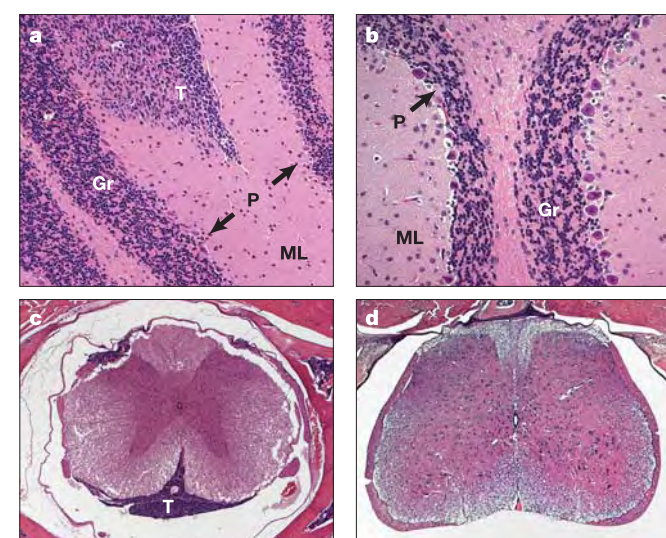


Figure 3 | Medulloblastoma pathology. Sections of an SB-induced medulloblastoma and control cerebellum, stained with haematoxylin and eosin. **a**, Section of the cerebellum from animal TG6057-17106 showing normal morphology, with the Purkinje cell layer (P) adjacent to the granule cell layer (Gr). Tumour cells (T) have invaded the molecular layer (ML). **b**, Comparable section for a normal cerebellum. **c**, Tumour (T) has grown down the brain stem adjacent to the spinal cord. **d**, Comparable section for a normal spinal cord.

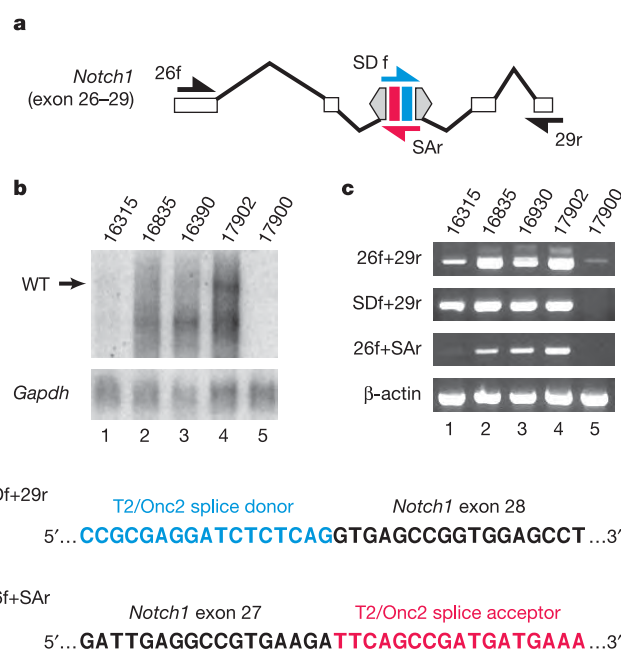


Figure 4 | Analysis of *Notch1* integrations. **a**, Structure of mutated *Notch1* allele in SB-induced T-cell leukaemias. White, exons; grey, transposon IRDRs; red, transposon splice acceptor; blue, splice donor; arrows, primer binding sites (f, forward; r, reverse). **b**, Northern analysis using a 3' *Notch1* cDNA probe showed that all tumours with *Notch1* integrations (lanes 1–4) express a truncated *Notch1* transcript. The transcript in tumour 16315 is less intense but can be seen on longer exposure. WT, wild type. **c**, RT-PCR shows that only tumours with *Notch1* integration express a truncated *Notch1* transcript. The sequence of the SB-*Notch1* splice junction in tumours is shown below.

3' end of *Notch1* (Fig. 4b, c). This fusion transcript mimics that seen in human T-ALL patients with t(7;9), in which the translocation drives expression of an activated *NOTCH1* carboxy-terminal protein fragment²⁰. Furthermore, transgenic mice overexpressing a similar fragment of *Notch1* develop T-cell lymphoma²¹. These results confirm that SB-induced tumours are induced by insertional mutagenesis.

Cooperating cancer genes and pathways

Among the six tumours with activating integrations at *Notch1*, three also had activating integrations upstream of *Rasgrp1* (Fig. 5, Supplementary Table 4), a gene that positively regulates *Ras* signalling. The probability of finding two tumours with integrations in the same two pair of genes by chance is low ($P = 9.2 \times 10^{-5}$; Supplementary Methods). Integrations in *Rasgrp1* were seen only in tumours with *Notch1* integrations, indicating that *Ras* signalling might cooperate with *Notch1* in tumour induction. Two tumours with *Notch1* and *Rasgrp1* integrations also had activating integrations upstream of *Sox8*, an uncharacterized member of the Sox family of SRY-related high-mobility-group (HMG)-box DNA-binding proteins (Fig. 5). The probability of finding two tumours with integrations in *Notch1*, *Rasgrp1* and *Sox8* by chance is exceedingly low ($P = 2.2 \times 10^{-7}$) and indicates that *Sox8* could represent another signalling pathway that cooperates with *Notch1* in tumour induction. Finally, two *Notch1* tumours also have activating integrations upstream of *Runx2* (Fig. 5), indicating that *Runx2* might represent yet another *Notch1*-cooperating gene.

Although most genes mutated by SB transposition in tumours were identified in only one tumour, several belong to related

signalling pathways. Careful annotation identified seven pathways that were commonly disrupted in SB-induced tumours (Supplementary Table 6). Similar analysis of the integration sites cloned from embryos did not reveal any similar trends. Integrations in most cases are predicted to affect a given pathway in a similar manner, but they accomplish this through the disruption of different genes. For example, six tumours have transposon-induced mutations that are predicted to result in decreased TNF signalling. Similarly, decreased rates of receptor recycling, increased signalling through the Ras superfamily, increased Jak/Stat signalling and increased Wnt signalling are all common pathways affected by SB transposition in tumours (Supplementary Table 6). The identification of genes and signalling pathways that cooperate to induce cancer will make it possible to develop better combinatorial therapies for treating human cancer.

Discussion

We describe here a non-viral insertional mutagen that efficiently induces tumours in wild-type mice. SB transposition can easily be controlled to mutagenize a specific target tissue by simply restricting the site of SB transposase expression. In principle, SB transposition can be adapted to generate virtually any kind of cancer by restricting the sites or timing of transposase expression. This should also reduce or eliminate the embryonic death observed in our studies, in which the SB transposase was widely expressed. The high frequency of transposition possible with our system might also prove valuable in generating tumours in other cell types and in wild-type genetic backgrounds that do not carry a predisposing cancer mutation. This will make it possible to model various types of human cancer without any knowledge of the causative events and in a more unbiased manner. Together with high-throughput LM-PCR, cancer genes and their pathways associated with tumorigenesis can be rapidly identified, providing insight into human cancer through the use of mouse models. Given the unexpectedly high somatic SB transposition frequencies achieved in our studies, there is no theoretical reason why SB transposition frequencies cannot be increased in the mouse germ line to levels that would permit efficient forward genetic screens with SB. Because SB tags the mutated gene, the gene is much easier to clone than one mutated by a point mutagen such as ethylnitrosourea. Finally, the uses of SB are also not restricted to the mouse. SB was originally isolated from fish and has already been shown to function in zebrafish²² and medaka²³. SB could therefore be used in forward genetic screens in any higher eukaryote in which transgenesis is possible.

METHODS

Generation of the RosaSB allele. An expression cassette consisting of an *En2* splice acceptor, SB11 cDNA and SV40 poly(A) was cloned upstream of a floxed PGKneo cassette. This cassette was then recombined into a plasmid containing a TK selection cassette as well as the promoter region, exon 1 and a portion of the single intron of the *Rosa26* locus by using the recombineering strategy described previously²⁴. This recombination introduced the knock-in cassette into the *XbaI* site of the *Rosa26* intron that has been used in previous *Rosa26* knock-in alleles²⁵. The targeting plasmid was then linearized and introduced into ES cells. After selection, ES cell colonies were picked and DNA was extracted and digested with *SpeI* to screen the 5' region of *Rosa26* and *BglI* for the 3' region. Southern blotting was performed on the 5' region with the use of a 908-bp *SacI* fragment of the *Rosa26* promoter region. A 667-bp *SspI* fragment derived from the intron of *Rosa26* was used to confirm the 3' recombination site by Southern blot analysis. Three independent clones were injected into blastocysts to derive three RosaSB knock-in lines. Mice were genotyped by PCR with primers specific for the SB11 cDNA: 5'-ATGGGAAATCAAAGAAATCAGCCAAG-3' and 5'-GCCAAA CAGTTCATTATTTGTTTCATCAGACCA-3'. One line was subsequently maintained by backcrossing to C57BL/6 mice.

Generation of T2/Onc2 transgenic mice. The T2/Onc2 transposon was made by replacing the *HpaI/BglII* fragment containing the *En2* splice acceptor from pT2/Onc with a fragment containing a larger portion of the *En2* exon. In addition to this change, the overall size of T2/Onc2 was reduced (2,050 bp, compared with 2,163 bp for T2/Onc) but was otherwise identical to T2/Onc. The

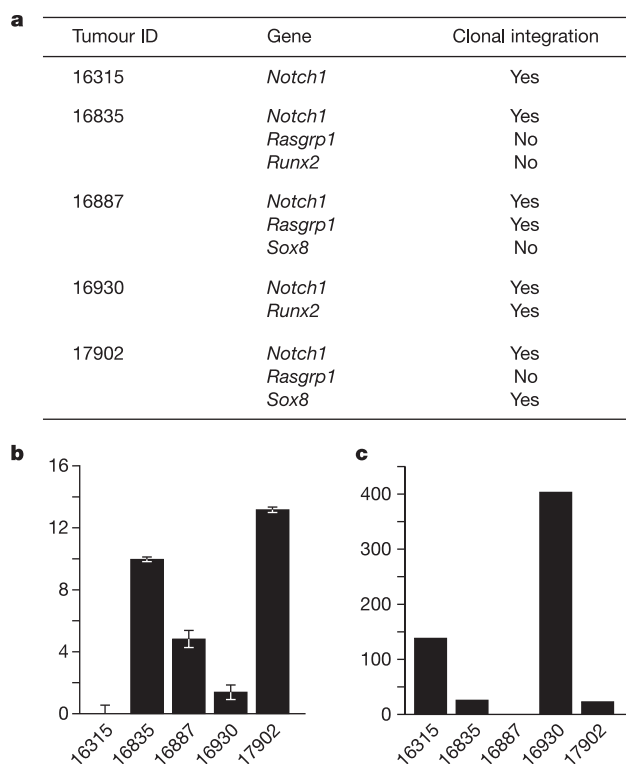


Figure 5 | *Notch1* cooperating genes. **a**, Clonality of *Notch1*, *Rasgrp1*, *Sox8* and *Runx2* integrations in *Notch1* tumours was determined by Southern analysis. **b**, **c**, Quantitative PCR was used to measure the expression levels of *Rasgrp1* (**b**) and *Runx2* (**c**) in tumours relative to a glyceraldehyde-3-phosphate dehydrogenase gene, *Gapdh* control. Results are means $\pm$ s.d. for three independent assays. Error bars in **c** are too small to show. Quantitative PCR could not be reliably performed on *Sox8*; *Sox8* expression in tumours with *Sox8* integrations was therefore monitored by RT-PCR (data not shown).

pT2/Onc2 plasmid was linearized using *ScaI* and prepared for microinjection into (B6C3)_F₂ hybrid embryos with standard techniques. Tail biopsy DNA from founder animals was screened by Southern blotting using an *En2* splice acceptor probe. Transgenic lines were established by crossing to C57BL/6. Offspring were genotyped by PCR using primers 5'-CAGTTGAAGTCGGAAGTTTA-3' and 5'-GGAATTGTGATACAGTGAAT-3'.

Calculation of expected number of common sites. A Java program was created to simulate the random SB transposon insertions in the mouse genome. The program randomly selected 491 (number of sites cloned from embryos) or 782 (number of sites cloned from tumours) TA motifs from the whole mouse genome by using a random number generator. The program then counted the number of common integration sites by calculating distances between the integration sites. After repetition of this procedure 10,000 times, the average expected number of common integration sites was determined.

Received 3 February; accepted 25 April 2005.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements We thank D. Swing and R. Koogler for generating the T2/Onc2 transgenic mice and maintaining all mouse strains, and E. Southon and S. Reed for generating mice carrying the RosaSB knock-in allele. This research is supported by the Department of Health and Human Services, National Institutes of Health and the National Cancer Institute.

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The discovery of a galaxy-wide superwind from a young massive galaxy at redshift $z \approx 3$

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High-velocity galactic outflows, driven by intense bursts of star formation and black hole accretion, are processes invoked by current theories of galaxy formation¹ to terminate star formation in the most massive galaxies and to deposit heavy elements in the intergalactic medium. From existing observational evidence^{2,3} (for high-redshift galaxies) it is unclear whether such outflows are localized to regions of intense star formation just a few kiloparsecs in extent, or whether they instead have a significant impact on the entire galaxy and its surroundings. Here we present two-dimensional spectroscopy of a star-forming galaxy⁴ at redshift $z = 3.09$ (seen 11.5 gigayears ago, when the Universe was 20 per cent of its current age): its spatially extended Ly α line emission appears to be absorbed by H I in a foreground screen covering the entire galaxy, with a lateral extent of at least 100 kpc and remarkable velocity coherence. This screen was ejected from the galaxy during a starburst several 10^8 years earlier and has subsequently swept up gas from the surrounding intergalactic medium and cooled. This demonstrates the galaxy-wide impact of high-redshift superwinds.

The formation of galaxies requires gas to cool in haloes of dark matter that collapse under gravity from the expansion of the Universe. However, cooling alone overproduces bright galaxies at the present day, so models incorporate thermal conduction, photoionization and galaxy merging, together with additional 'feedback'¹ in the form of galactic-scale outflows. The latter are powered by supernovae and massive stellar winds, or by relativistic winds and jets resulting from gas accretion onto supermassive black holes. Although starburst superwinds have been studied⁵ in local dwarf galaxies (such as M82), observational evidence for their counterparts in young massive galaxies at high redshift has been less direct.

In Lyman-break galaxies (LBGs)—high-redshift galaxies with moderate masses and star-formation rates—spectroscopic evidence points to powerful outflows^{2,3} of interstellar gas, with absorption lines being blueshifted by several hundred kilometres per second from the galaxy's systemic velocity. The gravitationally lensed LBG MS1512-cB58 at redshift $z = 2.73$ is a clear example³: absorbing gas is outflowing at $\sim 255 \text{ km s}^{-1}$ at a rate exceeding the star-formation rate, and although it covers the entire star-forming region, this shows only that the radius of any shell exceeds $\sim 1 \text{ kpc}$; it is not known whether the outflows are collimated and localized to star-forming regions, or whether they are galaxy-wide. The latter is suggested by the observed decrease in the intergalactic medium (IGM) H I opacity in background quasar sightlines that are close in projection to LBGs⁶ (within 0.7 Mpc co-moving). This may indicate that LBGs drive superwinds at $\sim 600 \text{ km s}^{-1}$ for several 10^8 yr , or it might be a statistical fluctuation since there are few LBG–quasar pairs at the smallest separations.

To characterize such outflows better via absorption studies, a background light source is needed with a spatial extent somewhat

larger than an LBG stellar body or a quasar sightline. Such a source is provided by the recently discovered Ly α -emitting 'blobs' (hereafter LABs), associated with LBGs in the SSA22 protocluster at redshift $z = 3.09$, a structure which is likely to evolve into a rich cluster of galaxies. With sizes $\sim 100 \text{ kpc}$ and Ly α luminosities $\sim 10^{44} \text{ erg s}^{-1}$, the LABs resemble the emission-line haloes of high-redshift radio galaxies, but their radio emission is much fainter than in typical high-redshift radio galaxies⁷. Models for LAB Ly α emission include superwind ejecta⁸ and cooling radiation from gravitationally heated gas⁹. Both LABs are submillimetre sources¹⁰, with LAB-1 the more powerful and consistent with $\sim 1,000 M_{\odot} \text{ yr}^{-1}$ of star formation. LAB-2 has an X-ray detection, suggestive of an obscured active galactic nucleus¹¹, whereas LAB-1 does not.

We recently observed the LAB Ly α haloes using integral-field spectroscopy, which, unlike conventional slit spectroscopy, gathers spatially resolved spectra over a two-dimensional area. Such information is essential for a complete picture of morphologically complex objects like the LABs. We used the SAURON Integral Field Spectrograph¹² on the William Herschel Telescope, providing moderate resolution spectroscopy ($\sim 4 \text{ \AA}$ full-width at half-maximum,

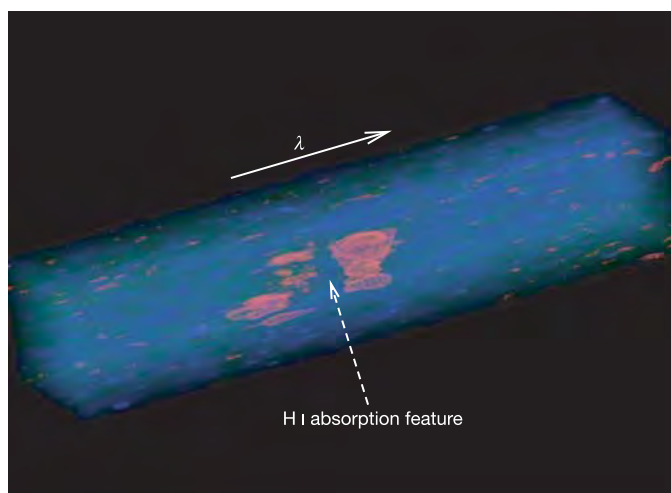


Figure 1 | Part of the data cube obtained from integral field spectroscopy of the $z = 3.09$ galaxy LAB-2, showing the Ly α emission line. The absorption feature, interpreted as due to foreground H I, is indicated. A data cube is a three-dimensional structure for spectroscopic data from integral field spectrographs. Here the long axis corresponds to the dispersion axis and the other two axes represent spatial dimensions on the sky, that is, the data cube stores a spectrum for each point in a two-dimensional region of sky. With visualization software, the data-cube representation can provide useful qualitative insights into complex data sets. λ , wavelength.

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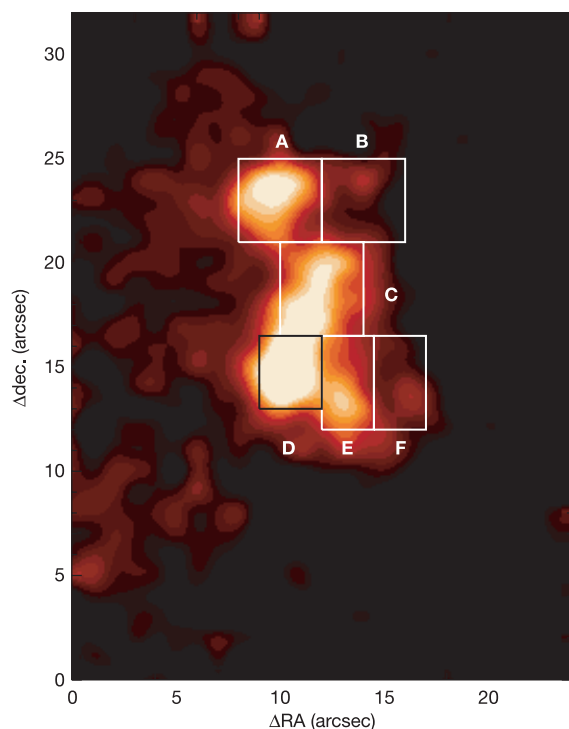


Figure 2 | A reconstructed image of the $z = 3.09$ galaxy LAB-2 in the $\text{Ly}\alpha$ emission line. This shows the extent of its gaseous halo (10 arcsec on the sky equates to a physical distance of 76 kpc in this galaxy with the cosmological parameters $H_0 = 70 \text{ km s}^{-1} \text{ Mpc}^{-1}$, $\Omega_M = 0.3$ and $\Omega_\Lambda = 0.7$). The image was derived from observations with the SAURON integral field spectrograph, by collapsing the data cube shown in Fig. 1 along the wavelength direction. The labels indicate regions for which one-dimensional $\text{Ly}\alpha$ emission line profiles have been extracted, as shown in Fig. 3. The underlying $\text{Ly}\alpha$ emission is likely to be from gas in the halo of the associated Lyman-break galaxy⁴ (at position C), arising from a combination of superwind ejecta⁸, cooling radiation⁹, and gas photoionized by the obscured active nucleus¹¹.

FWHM) over a $41 \text{ arcsec} \times 31 \text{ arcsec}$ area, sampled with 0.95-arcsec lenslets. LAB-2 was observed for 15 h (in thirty 30-min sub-exposures), but unlike LAB-1¹³ exhibits simple structure in $\text{Ly}\alpha$. At most positions the emission line is double-peaked, as shown in Fig. 1 and in the line profiles of individual regions (Figs 2 and 3). The position of the profile's central minimum varies across the galaxy by less than 60 km s^{-1} in its rest-frame. The observing strategy of dithering between sub-exposures ensures that this feature is not a charge-coupled device (CCD) detector defect, nor is it due to a night-sky emission feature. The simplest interpretation is that part of the $\text{Ly}\alpha$ emission is scattered out of our line of sight by neutral hydrogen in a coherent foreground screen.

The line profiles were each modelled with a gaussian emission line and a superimposed Voigt profile absorber (all convolved with the instrumental response), as in Fig. 3. The H I column densities are $N_{\text{H I}} \approx 10^{19} \text{ cm}^{-2}$, and the relative amplitudes of the red and blue line wings reflect velocity differences between the emission and absorption components. The absorber is typically blueshifted from the underlying peak by less than 250 km s^{-1} ; the emission shows a range in absolute velocity of $\sim 290 \text{ km s}^{-1}$, with individual line-widths of $\sim 1,000 \text{ km s}^{-1}$ FWHM. At position B, no absorber is detected, perhaps because the underlying emission is more blueshifted than elsewhere.

An absorbing shell is predicted to form when a starburst-heated hot gas bubble becomes over-pressurized relative to the interstellar medium and hence breaks out of the galactic disk, accelerates, and

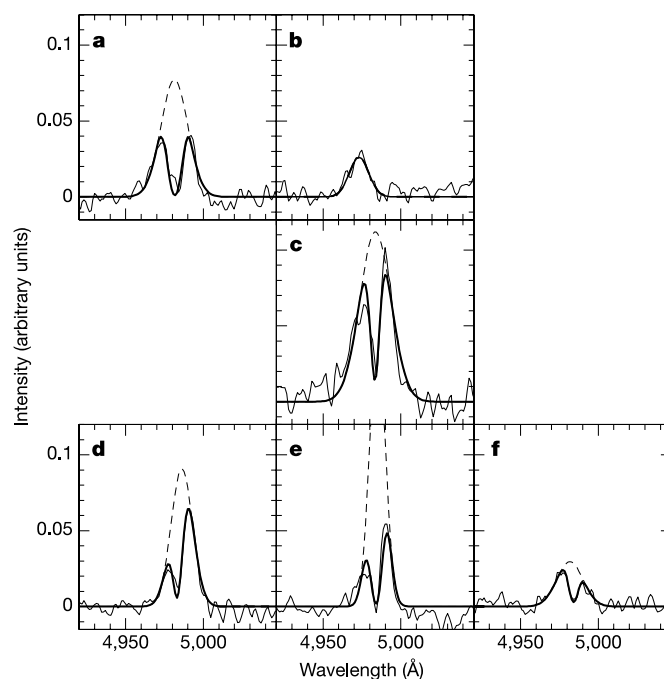


Figure 3 | $\text{Ly}\alpha$ emission line profiles across the galaxy LAB-2 at $z = 3.09$.

A profile is shown for each region labelled in Fig. 2. Thick solid lines are fits to a model where the intrinsic $\text{Ly}\alpha$ emission (dashed lines) is partially absorbed by foreground H I . The measured H I column densities are $\sim 10^{19} \text{ cm}^{-2}$, although the modest spectral resolution implies 1σ uncertainties of at least a factor of ~ 10 , so the column density may not be uniform across the galaxy. The widths of the absorbers (expressed as the gaussian 'b-parameter' in the Voigt profile) are $\sim 20 \text{ km s}^{-1}$, which for thermal broadening implies a kinetic temperature $\sim 10^4 \text{ K}$ (as expected on photoionization grounds and consistent with the lack of bulk motion across the shell). Note, however, that similar $\text{Ly}\alpha$ profiles can result from a $\text{Ly}\alpha$ source in an H I slab of much higher optical depth¹⁹; the twin peaks then arise because the escaping photons scatter into the line wings where the optical depth is lower. This would not, however, explain why the apparent absorption feature is constant in wavelength while the fitted emission velocity varies by almost 300 km s^{-1} .

fragments as a result of Rayleigh–Taylor instabilities. Hot gas then escapes into the halo and forms a second shell of swept-up IGM. Evolutionary models for the $\text{Ly}\alpha$ emission from such superwinds¹⁴—which are consistent with Hubble Space Telescope observations of local starburst galaxies¹⁵—suggest that the LAB-2 absorber is in a late phase, where the shell has cooled and slowed sufficiently to absorb the underlying emission. Figure 4 illustrates the model.

Because the absorbing shell's transverse extent is at least 100 kpc, it is probably a comparable distance in front of the $\text{Ly}\alpha$ source (although the constant absorber velocity implies that, if spherical, the radius must be considerably larger). At a velocity of $1,000 \text{ km s}^{-1}$, it takes 10^8 yr to travel 100 kpc. Although $1,000 \text{ km s}^{-1}$ is the expected level of the initial velocity, it has since slowed owing to mass loading from the IGM and work against the galaxy's gravity; this implies an age of several 10^8 yr .

We now consider the cooling of the shocked gas. Gas behind a shock of speed v_{shock} is heated to $T = 1.4 \times 10^7 (v_{\text{shock}}/1,000 \text{ km s}^{-1})^2 \text{ K}$, from which the radiative cooling time is $t_{\text{cool}} = 1.5 \text{ kT}/4n_{\text{halo}}\Lambda$, where Λ is the cooling function¹⁶ ($> 5 \times 10^{-23} \text{ erg cm}^3 \text{ s}^{-1}$ for 10^6 – 10^7 K and solar abundances) and n_{halo} is the surrounding gas density (the factor 4 reflects the density increase behind a strong shock). Together, these imply that $t_{\text{cool}} < 4.6 \times 10^5 \text{ yr} (v_{\text{shock}}/1,000 \text{ km s}^{-1})^2 / (n_{\text{halo}}/\text{cm}^{-3})$. Moving out from the galaxy into the IGM at 100 kpc, the density n_{halo} will range from a few 10^{-3} to a few 10^{-4} cm^{-3} , so for shock

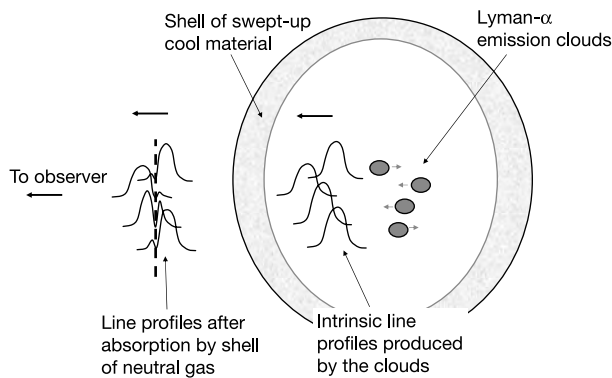


Figure 4 | An illustration of our model for the Ly α emission and absorption in the redshift $z = 3.09$ galaxy LAB-2. The Ly α emission is produced by gas with a velocity dispersion $\sim 1,000 \text{ km s}^{-1}$ and bulk motions $\sim 290 \text{ km s}^{-1}$. This emission is then absorbed/scattered by H I in a foreground shell. The shell's velocity coherence across the source suggests that it is over 100 kpc from galaxy, having been swept up from the surrounding medium by a starburst-driven outflow several 10^8 yr earlier. The possibility that the absorption is not directly associated with LAB-2, but part of a larger foreground absorber which just happens to cover it, is unlikely since this would imply an overall size much larger than absorbers of comparable $N_{\text{H I}}$ in quasar sightlines²⁰: the LAB-2 absorber column density is between that of a Lyman-limit system (diameter $\sim 80 \text{ kpc}$) and that of a damped Ly α absorber ($\sim 30 \text{ kpc}$). Such sizes are consistent with recent modelling²¹ and cosmological simulations²². Moreover, it has been shown²³ that all damped Ly α absorbers at redshift $z \approx 3$ with $N_{\text{H I}} > 2 \times 10^{20} \text{ cm}^{-2}$ can be explained by LBG galactic winds, if they are driven out to $\sim 27 \text{ kpc}$.

velocities of several hundred kilometres per second, the shocked gas can cool within the expansion timescale of a few 10^8 yr . The gas is expected¹⁷ to cool to $\sim 10^4 \text{ K}$ and come into photoionization equilibrium with the metagalactic ultraviolet radiation field, with an H I fraction ~ 0.1 – 0.001 . Indeed, for the above n_{halo} values, the total column density swept up out to 100 kpc could be up to 10^{21} cm^{-2} , compared with the measured $N_{\text{H I}} \approx 10^{19} \text{ cm}^{-2}$. The implied mass of the shell, if it covers an area 100 kpc square, could be almost $10^{11} M_{\odot}$. For a velocity of 250 km s^{-1} , the kinetic energy of such a shell is $6.3 \times 10^{58} \text{ erg}$, which we can compare with the energetics of supernovae: the LBG associated with LAB-2 (M14) has a near-infrared K_s-band magnitude⁴ of 21.7, which implies (from galaxy evolution models¹⁸) a baryonic mass of $\sim 10^{11} M_{\odot}$, mostly in stars. For a normal stellar initial mass function, supernovae provide $\sim 10^{49} \text{ erg}$ per solar mass of stars¹, so $\sim 10^{60} \text{ erg}$ is available and the superwind model appears energetically feasible.

We thus have a consistent picture in which the Ly α absorber is a highly ionized shell of gas swept up from the IGM over several 10^8 yr since the end of the starburst, having slowed to within a few hundred kilometres per second of the galaxy's systemic velocity. This demonstrates that superwinds are a galaxy-wide phenomenon, whose impact is not confined to small-scale regions of current star formation, as previous observations might have suggested. This underscores their importance as a primary feedback agent in galaxy formation.

Received 6 December 2004; accepted 5 May 2005.

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Acknowledgements We thank the SAURON team for supporting this programme, together with E. Emsellem, E. Jourdeuil and ING staff for support on La Palma. The construction of SAURON was financed by contributions from ASTRON/NWO, the Institut des Sciences de l'Univers, the Université Claude Bernard Lyon-I, the universities of Durham and Leiden, The British Council, PPARC, and NOVA. R.J.W. and R.G.B. acknowledge support from PPARC, and R.G.B. also thanks the Leverhulme Foundation. J.G. is supported by the Euro3D research training network.

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LETTERS

An extrasolar giant planet in a close triple-star system

Maciej Konacki¹

Hot Jupiters are gas-giant planets orbiting with periods of 3–9 days around Sun-like stars. They are believed to form in a disk of gas and condensed matter at or beyond ~ 2.7 astronomical units (AU—the Sun–Earth distance) from their parent star^{1,2}. At such distances, there exists a sufficient amount of solid material to produce a core capable of capturing enough gas to form a giant planet. Subsequently, they migrate inward to their present close orbits³. Here I report the detection of an unusual hot Jupiter orbiting the primary star of a triple stellar system, HD 188753. The planet has an orbital period of 3.35 days and a minimum mass of 1.14 times that of Jupiter. The primary star's mass is 1.06 times that of the Sun, $1.06 M_{\odot}$. The secondary star, itself a binary stellar system, orbits the primary at an average distance of 12.3 AU with an eccentricity of 0.50. The mass of the secondary pair is $1.63 M_{\odot}$. Such a close and massive secondary would have truncated a disk around the primary to a radius of only ~ 1.3 AU (ref. 4) and might have heated it up to temperatures high enough to prohibit giant-planet formation^{5,6}, leaving the origin of this planet unclear.

The most massive short-period planets (orbital period $P_{\text{orb}} < 40$ d) orbit stars from binary stellar systems^{7,8}. Altogether, among ~ 130 extrasolar planets⁹ discovered with the spectroscopic precision radial velocity (RV) technique^{10,11}, over 20 belong to stars that also have stellar companions⁸. It is quite surprising, given that binary stars with separations smaller than ~ 2 arcsec have been excluded from the surveys. The stars from binary stellar systems known to harbour planets have a distant (widely separated in the sky) and/or faint stellar companion that does not contaminate the primary star spectrum and makes precision RV measurements much easier. The theories of planet formation in binary or multiple stellar systems are still at early stages, but such planets once formed would enjoy a wide range of dynamically stable orbits^{12,13}. The subject

of planet formation in binary stellar systems is an important issue, and not only because the frequency of binaries among field stars older than 1 Gyr is $\sim 60\%$ (ref. 14) and is even higher among pre-main-sequence stars¹⁵. If we believe that the same basic processes lead to the formation of planets around single stars and components of binary

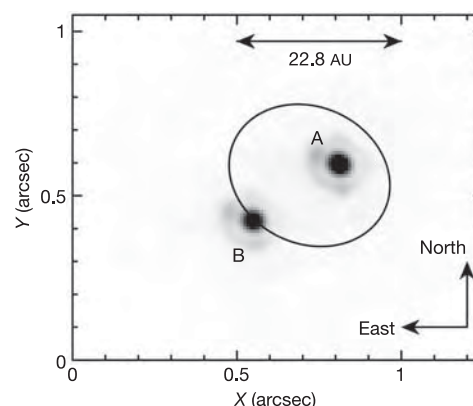


Figure 1 | Adaptive optics image of HD 188753. The image was taken with the NIRC2 camera at the Keck-II telescope on 25 March 2005 (MJD = 53454.65813) in the K band (K_s , $\lambda = 2.146 \mu\text{m}$). HD 188753 was also observed in the H ($\lambda = 1.633 \mu\text{m}$) and J ($\lambda = 1.248 \mu\text{m}$) bands to obtain the brightness ratios of the primary to the secondary (Table 2). Only the central part of the image is shown, as the entire field of view is 10 arcsec. The size of the slit used with HIRES is 0.574×3.5 arcsec. The scale of the image is 9.9 mas per pixel, which corresponds to 0.45 AU per pixel at the distance of HD 188753. The position of the secondary with respect to the primary is $\rho = 313.16 \pm 0.42$ mas, $\theta = 123^\circ.16 \pm 0.06^\circ$; ρ is the length of the separation vector AB, and θ is the position angle of that vector measured from north through to east. The semi-major axis of the stellar companion to the secondary is ~ 1.5 pixels. The solid line represents the most up-to-date orbit of the pair AB, determined on the basis of Hipparcos data and almost a century of archival astrometry²². The apparent semi-major axis is 0.27 arcsec, the orbital period is 25.7 yr, the orbital inclination is 34° and the eccentricity is 0.50. Their formal 1σ errors are not available in literature, but are not larger than a few per cent (ref. 22). This orbit is grade 1 ('definitive') in the 'Sixth Catalog of Orbits of Visual Binary Stars' (<http://ad.usno.navy.mil/wds/orb6.html>). As one can see, the orbit is indeed very good as the predicted position of the secondary agrees very well with the observed one. I adopt a conservative error estimate of 10% for the eccentricity, as its values available in literature range from 0.52 (1927) to 0.47 (1990) and 0.50 (1999). At the 3σ level in the K band, I can exclude any additional companions (physically associated with the system or chance alignments) in the entire field of view to a magnitude difference of $\Delta m = 5.0$ compared to the primary. They might have 'hidden' in front of or behind the two stars, but the probability of such an alignment is only 0.015%, for a randomly placed star in the 10 arcsec field of view. Therefore, the spectrum of HD 188753 is probably not contaminated by any other sources.

Table 1 | HD 188753 AB radial velocities

Date (HJD – 2400000)	RV _A (km s ^{–1})	Error (km s ^{–1})	RV _B (km s ^{–1})	Error (km s ^{–1})
52863.91581	–22.131	0.0254	–14.785	0.0430
52961.73546	–21.935	0.0206	–36.133	0.0451
52962.71782	–21.762	0.0195	–36.182	0.0595
53095.12668	–22.101	0.0253	–32.810	0.0351
53205.86331	–22.207	0.0202	–10.566	0.0553
53276.75041	–22.238	0.0187	–34.406	0.0398
53277.81261	–22.168	0.0308	–34.017	0.0728
53328.70280	–22.526	0.0193	–14.014	0.0458
53328.75964	–22.510	0.0187	–14.116	0.0539
53329.70192	–22.572	0.0185	–13.511	0.0511
53329.75728	–22.512	0.0182	–13.547	0.0572

The radial velocities of the primary (RV_A) and the secondary (RV_B) spanning 466 d and their 1σ errors in the barycentric reference frame. Larger velocity errors for the secondary which is two times fainter than the primary are typical for the velocities derived with the cross-correlation technique.

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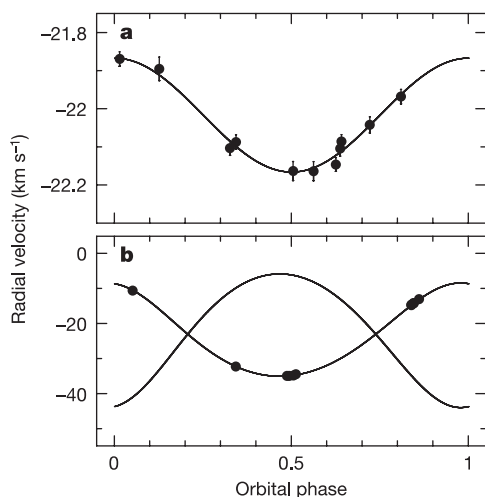


Figure 2 | Radial velocities as functions of orbital phase. Radial velocity (RV) of the primary (a) and the secondary (b) and their 1σ errors as a function of the orbital phase. The best-fit models are denoted with the solid line. Note that the orbital period of the primary (A) is 3.35 d and that of the secondary (B) is 156 d. The long-term RV variations due to the orbital motion of the pair AB ($P_{\text{orb}} = 25.7$ yr) have been subtracted (Table 2). The best-fit r.m.s. (root mean square of the post-fit residuals) is 14.7 m s^{-1} for the primary ($\chi^2 = 1.11$ with 5 degrees of freedom) and 83.3 m s^{-1} ($\chi^2 = 4.49$ with 4 degrees of freedom) for the secondary. However, the r.m.s. for the secondary is affected by one RV measurement taken in poor weather conditions (HJD=2453277.81261). If this point is not included in the fit, the r.m.s. is 42.2 m s^{-1} ($\chi^2 = 1.96$ with 3 degrees of freedom). The second solid line in b denotes the expected RV modulation of the stellar companion to the secondary. Its spectral lines cannot be detected, as the estimated brightness ratio (from the isochrones, see Table 2) between the primary and the stellar companion to the secondary is ~ 24 or more. Error bars in b are smaller than the size of the data points.

stars, then such processes should be equally feasible regardless of the presence of stellar companions. Therefore planets from close binary stellar systems may supply strong constraints on planet formation scenarios in general.

The molecular iodine (I_2) absorption cell technique¹¹ is the most common way to obtain precision RV observations. In this technique a star is observed through an I_2 gas cell, which superimposes a dense set of narrow absorption lines onto a stellar spectrum and provides a fiducial wavelength scale against which the Doppler shifts and hence velocities are measured. The I_2 technique as currently practised fails for spectroscopic binary stars. However, I have developed a novel technique employing an I_2 absorption cell that enables me to accurately determine RVs of both components of double-lined binaries¹⁶ (Supplementary Information). It is now possible to obtain precision RVs of binary stars with components that have comparable brightness (and hence comparable mass) and an arbitrarily small separation. I now routinely achieve $10\text{--}30 \text{ m s}^{-1}$ RV precision for binary stars with spectral types up to F3. For later type binaries (G-K), the precision approaches $\sim 10 \text{ m s}^{-1}$. With this technique, I initiated in 2003 a survey for planets in binary and multiple stellar systems with the high-resolution echelle spectrograph (HIRES)¹⁷ on the Keck I 10-m telescope at the W. M. Keck Observatory (Hawaii).

HD 188753 was established to be a binary system in 1895 (ref. 18). Later it was determined that the secondary is itself a single-lined spectroscopic binary with an orbital period of ~ 155 days (ref. 19). The pair AB has a period of 25.7 yr, a semi-major axis a of 12.3 AU and an eccentricity e of 0.50 (see Fig. 1). From August 2003 to November 2004, I observed HD 188753 with HIRES/Keck-I. The 22 RVs (Table 1) reveal the presence of a giant planet orbiting the primary with a period of 3.3481 ± 0.0009 d, and allow for an improved orbital solution for the secondary pair (Fig. 2). In order to verify that the RV variations of the primary are not due to surface magnetic activity, I examined Ca II H (wavelength $\lambda = 396.8$ nm) absorption

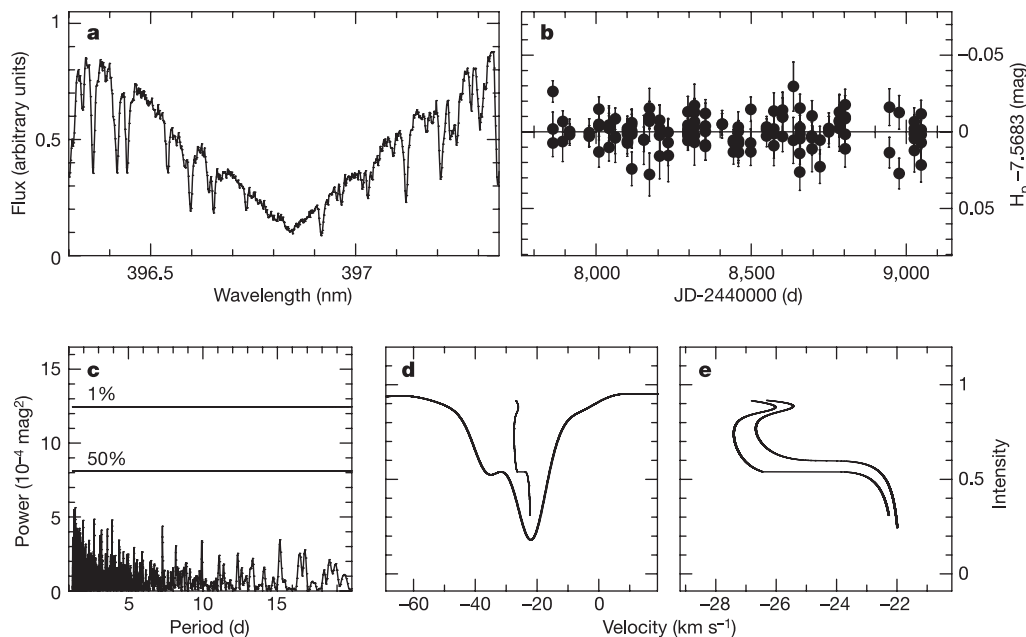


Figure 3 | Spectroscopy and photometry of HD 188753. a, A representative region around the Ca II H ($\lambda = 396.8$ nm) absorption line. There is no trace of any emission features in the middle of the line, indicating low chromospheric activity. All collected spectra show no indication of such activity. b, The Hipparcos photometry (H_p , around the median value)²⁰ as a function of time. Error bars are 1σ . c, The periodogram of the photometric time series, indicating no detectable periodicity. The false alarm probabilities of 50% and 1% are denoted with solid lines. d, A one-dimensional cross-correlation function (CCF) of HD 188753

(HJD=2452961.73546) and a synthetic template spectrum. CCFs are typically used for single stars to determine the bisectors of spectral lines (that is, the shape indicators of spectral lines; the solid line in the middle of the CCF) and their possible variations that may mimic RV modulations. For a binary star like HD 188753, such bisectors have little use because of the partial blending of the components' spectral lines. e, 'Bisectors' for two consecutive nights HJD=2452961.73546 (left curve) and HJD=2452962.71782 (right curve) where the orbital phase of the planet changes from 0.722 to 0.015. There is no unusual change in their shape.

Table 2 | Orbital and physical parameters of HD 188753 AB and its companions

Parameter	HD 188753A	HD 188753B
Velocity amplitude (km s^{-1})	0.149 ± 0.012	13.3 ± 0.2
Orbital period (d)	3.3481 ± 0.0009	156.0 ± 0.1
Time of periastron (HJD)	2453146.81 ± 0.05	2453198 ± 5
Eccentricity	0.0 (assumed)	0.1 ± 0.03
Longitude of periastron (deg.)	NA	10 ± 11
Velocity offset (km s^{-1})	-22.017 ± 0.017	-23.1 ± 0.2
Linear trend in velocity ($\text{km s}^{-1} \text{d}^{-1}$)	$(-1.46 \pm 0.05) \times 10^{-3}$	$(5.2 \pm 0.25) \times 10^{-3}$
Quadratic trend in velocity ($\text{km s}^{-1} \text{d}^{-2}$)	$(-4.75 \pm 0.45) \times 10^{-6}$	NA
Companion distance from star (AU)	0.0446 ± 0.0010	0.67 ± 0.01
Star mass ($M_{\odot}$)	1.06 ± 0.07	0.96 ± 0.05
Stellar companion mass ($M_{\odot}$)	NA	0.67 ± 0.05
Planet mass ($m \sin i$, in M_{Jup})	1.14 ± 0.10	NA
Temperature (K)	$5,750 \pm 100$	$5,500 \pm 100$
Projected rotational velocity (km s^{-1})	2 ± 1	2 ± 1
Age (Gyr)	5.6 ± 3.3	5.6 ± 3.3
$K_{2\text{MASS}}$ (mag)	6.183 ± 0.020	6.393 ± 0.020
$J_{2\text{MASS}}$ (mag)	6.555 ± 0.021	6.909 ± 0.021
$H_{2\text{MASS}}$ (mag)	6.273 ± 0.026	6.512 ± 0.026

HD 188753 is a nearby and bright star (distance $d = 45.66 \pm 1.25$ pc, V-band magnitude $V = 7.43$ mag)²². The temperatures and rotational velocities of its components were derived by modelling the high signal-to-noise spectra with numerical model atmospheres²⁹. The best match to the observed composite spectra of HD 188753 was obtained for solar metallicity and $\log g = 4.5 \text{ cm s}^{-2}$, where g is surface gravity. The stars' masses, the age and their 1σ errors were derived using: the theoretical isochrones³⁰, the distance to the star and the magnitude difference ($\Delta m_{\text{H}} = 0.75$) from Hipparcos²², the 2MASS photometry of the binary and the respective brightness ratios of the components AB from the Keck-II/NIRC2 adaptive optics data (Fig. 1). The total mass of the system, $2.69 \pm 0.10 M_{\odot}$, agrees well with the total mass derived from the Hipparcos astrometry, $2.73 \pm 0.33 M_{\odot}$ (ref. 22). From the available data, one can also derive the orbital inclination for the secondary pair, $44^{\circ} \pm 4^{\circ}$. The orbital model of the radial velocity (RV) variations included linear ($a_1(t - T_0)$) and quadratic terms ($a_2(t - T_0)^2$) to account for the RV changes due to the orbital motion of the pair AB ($P_{\text{orb}} = 25.7$ yr, Fig. 1). For the orbit of the component B ($P_{\text{orb}} = 156$ d), I have only included a linear term since the orbital phase coverage is not yet sufficient to obtain a robust orbital solution. The uncertainties of the orbital elements are the formal least-squares 1σ errors. The error for the planet mass and the companions' semi-major axes reflect my estimates of uncertainties in the orbital elements and the stars' masses. NA, not applicable.

lines as well as the Hipparcos photometric data²⁰ and found no indication of such activity (see Fig. 3). With high confidence I can also rule out that these RV variations are caused by a faint spectroscopic binary, which could contaminate the spectrum of HD 188753 by being accidentally along the same line of sight (see Figs 1 and 3). From the spectroscopy, 2MASS photometry²¹ and the Hipparcos distance to the star²², one can derive the physical parameters of the system (see Table 2). The lower limit on the planet mass, $m \sin i$, where i is the orbital inclination angle, is $1.14 \pm 0.10 M_{\text{Jup}}$, where M_{Jup} indicates the mass of Jupiter. The orbital inclination of the planet would have to be 5° or less for the planet to be in the brown dwarf regime ($m > 13 M_{\text{Jup}}$). For a random distribution of the orbital inclination angles, the probability of such a low value is only 0.38%. Assuming that the planet has the same orbital inclination as HD 188753 AB (34° ; ref. 22), its mass would be $2.04 M_{\text{Jup}}$.

The planet around HD 188753A falls into the category of hot Jupiters — giant planets with orbital periods of ~ 3 –9 d. Like many other hot Jupiters from binary or multiple stellar systems, it has a larger mass than a typical hot Jupiter orbiting a single star. Otherwise, this system is unique. The semi-major axis of the pair AB is only 12.3 AU, whereas the three closest binary stars with extrasolar planets (HD 41004, Gl 86 and γ Cep) have semi-major axes of ~ 20 AU or more^{23–25}. Moreover, the primary star (the brightest one in the system) harbouring the planet is less massive than the secondary pair. The total mass of the secondary and its stellar companion is $1.63 M_{\odot}$, whereas the primary has a mass of $1.06 M_{\odot}$ (Table 2). The primaries with planets in HD 41004, Gl 86 and γ Cep are more massive than their stellar secondaries by a factor of three or more.

HD 188753 constitutes a very challenging case for current theories of the formation of giant planets. It is commonly accepted that hot Jupiters are formed far away from their parent star and subsequently migrate inward to their present close orbits³. In this scenario, the formation occurs beyond the 'snowline' — the minimum distance from a star at which ice grains can condense. The snowline is typically placed at or beyond ~ 2.7 AU (refs 1, 2). However, it is known that the secondary can significantly truncate a protoplanetary disk around the primary²⁶. For a system like HD 188753 ($q = M_1/(M_1 + M_2 + M_3) = 0.39$, where q is the ratio of the primary star mass to the total mass of the triple system and M_1 , M_2 and M_3 are the masses of the three stars in the system; $e = 0.50$,

$a = 12.3$ AU), the radius of a protoplanetary disk would be only ~ 1.3 AU (ref. 4), far less than a typical distance to a snowline. Even adopting a conservative estimate of the eccentricity error (10%, see Fig. 1), this radius is unlikely to be larger than ~ 1.75 AU at the 3σ level. Yet, the planet resides well within a stability zone that extends up to ~ 1.5 AU (ref. 13) from the parent star.

It appears that within the favoured scenario for the formation of hot Jupiters, the planet around HD 188753A could not be formed (unless for some reason the orbit of HD 188753 AB was very different during a planet formation phase). One possible explanation is that the snowline can indeed be as close as ~ 1 AU from the star¹. Another possibility is that hot Jupiters form *in situ*²⁷, near their current orbital locations. However, the problem is probably more challenging. It has been suggested that planet formation in binary systems may be less efficient because the stirring induced by the secondary can significantly heat up the protoplanetary disk⁵. This may hold true for both the standard core accretion scenario and the recently revived gravitational collapse scenario²⁸ of giant-planet formation⁶. Presumably, the environment of HD 188753 is particularly prohibitive given a small semi-major axis and the high mass of the secondary.

Received 15 April; accepted 23 May 2005.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements M.K. thanks S. Kulkarni for support and guidance, D. Sasselov for discussions, and C. Gelino for acquiring the NIRC2 data. The data presented here were obtained at the W. M. Keck Observatory (operated by the California Institute of Technology, University of California, and NASA), which was made possible by financial support from the W. M. Keck Foundation. This publication makes use of data products from the Two Micron All Sky Survey, which is a joint project of the University of Massachusetts and the Infrared Processing and Analysis Center/California Institute of Technology, funded by NASA and the NSF. M.K. acknowledges support from NASA. Q5

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LETTERS

A frequency comb in the extreme ultraviolet

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Since 1998, the interaction of precision spectroscopy and ultrafast laser science has led to several notable accomplishments. Femto-second laser optical frequency ‘combs’ (evenly spaced spectral lines) have revolutionized the measurement of optical frequencies^{1,2} and enabled optical atomic clocks³. The same comb techniques have been used to control the waveform of ultrafast laser pulses, which permitted the generation of single attosecond pulses⁴, and have been used in a recently demonstrated ‘oscilloscope’ for light waves⁵. Here we demonstrate intra-cavity high harmonic generation in the extreme ultraviolet, which promises to lead to another joint frontier of precision spectroscopy and ultrafast science. We have generated coherent extreme ultraviolet radiation at a repetition frequency of more than 100 MHz, a 1,000-fold improvement over previous experiments⁶. At such a repetition rate, the mode spacing of the frequency comb, which is expected to survive the high harmonic generation process, is large enough for high resolution spectroscopy. Additionally, there may be many other applications of such a quasi-continuous compact and coherent extreme ultraviolet source, including extreme ultraviolet holography, microscopy, nanolithography and X-ray atomic clocks.

For more than three decades, laser physics and laser spectroscopy have evolved along two seemingly opposite and disconnected frontiers, pursued by different communities of researchers. In the field of high resolution spectroscopy, scientists have learned to build ultra-stable continuous wave (c.w.) laser sources and they have perfected spectroscopy of laser-cooled atomic systems to reach extremely high spectral resolution⁷. In the field of ultrafast science, researchers have demonstrated ever-shorter pulses from mode-locked laser systems and ways of amplifying such pulses to very high intensities⁸. By focusing intense amplified pulses into a gas, coherent radiation down to the X-ray region can be generated by high harmonic generation (HHG)⁹. Since this process requires very high intensities ($>10^{13}$ W cm⁻²), the available laser power had to be concentrated into a small number of ultrashort pulses per second, limiting repetition rates for HHG to typically a few kHz.

To overcome this limitation, we couple the driving laser pulses from the oscillator into a high finesse optical resonator that contains the nonlinear medium. Inside this resonator, the circulating power is enhanced by a factor P (the resonator finesse divided by π) so that it can drive the nonlinear process (HHG) inside a gas target. For c.w. lasers, resonators with a finesse exceeding 100,000 can be constructed¹⁰ and overall nonlinear conversion efficiencies approaching unity can be achieved¹¹. In the case of a mode-locked ultrafast laser, however, complying with the extra requirements discussed below is more difficult: the output spectrum of a mode-locked laser contains not just a single c.w. mode but a comb of such modes, with frequencies

$$\omega_n = n\omega_r + \omega_{\text{CE}} \quad (1)$$

Here ω_r is the pulse repetition frequency and ω_{CE} the carrier envelope

(CE) frequency¹². Both ω_r and ω_{CE} reside in the radio frequency domain, whereas the optical frequencies of the comb are given by ω_n . The two frequency regions are connected by virtue of the large integer $n = 10^5 \dots 10^6$ that enumerates the modes. An optical resonator for such radiation has to be simultaneously resonant for each mode ω_n that originates from the laser. This can be accomplished with a resonator of appropriate length and zero group velocity dispersion, whose CE frequency is matched to the laser^{13,14}. Rephrased into time domain language, coherent pulse addition in a resonator occurs if (1) the round trip time of the pulse is the same as the period of the laser, (2) the pulse envelope does not change its shape during one round trip, and (3) the carrier phase shift with respect to the envelope of the circulating pulse is the same per round trip as the pulse to pulse change from the laser. For increasing finesse, the stored pulse undergoes an increasing number of round trips and therefore dispersion compensation becomes more critical. Nevertheless, such cavities have been demonstrated to enhance nonlinear conversion for 100-fs pulses¹⁵, and recently a resonator for sub-50-fs pulse duration has been demonstrated to provide an enhancement factor of up to 70, resulting in pulse energies of more than 200 nJ (ref. 16).

Instead of dumping the power from the resonator as has been done in earlier experiments¹⁶, we have placed a low-density xenon gas target as the nonlinear medium for HHG inside such a resonator. In contrast to the usual HHG schemes, the power that is not converted into the extreme ultraviolet (XUV) after a single pass through the medium is ‘recycled’ and can contribute in subsequent passes, so that higher total conversion efficiencies than for conventional schemes can be expected. Moreover, it becomes possible to use the high power at the full repetition rate of the laser. As a result, in addition to the comb of odd laser harmonics that stem from the periodicity of the carrier wave in one pulse, a nested frequency comb with a spacing given by the repetition frequency originates from the periodic pulse train. If the HHG spectrum contains only frequency components that are the sum of the original frequencies from equation (1), as expected from energy conservation, then the XUV comb structure of the k th laser harmonic (that is, the sum of k laser photons) is given by

$$\omega'_n = n\omega_r + k\omega_{\text{CE}} \quad (2)$$

where the integer n now runs over k times larger values than in equation (1). With such a widely spaced frequency comb, single modes that are effectively c.w. lasers may be isolated for high resolution spectroscopy. If a mode ω_n of the comb is tuned into resonance with a two-photon transition, any pair $\omega_{n-\ell} + \omega_{n+\ell}$ (with integer ℓ) is simultaneously resonant. This allows the use of the entire (broadband) power of the comb while maintaining the linewidth of a single mode¹⁷.

Direct frequency comb spectroscopy has been applied to atomic resonances in the infrared^{18–20} and in the ultraviolet range²¹, and a resolution approaching the natural linewidth with a laser-cooled sample has been reached^{18,20}. At the same time, the frequency comb may be phase coherently linked to a Cs atomic clock for accurate

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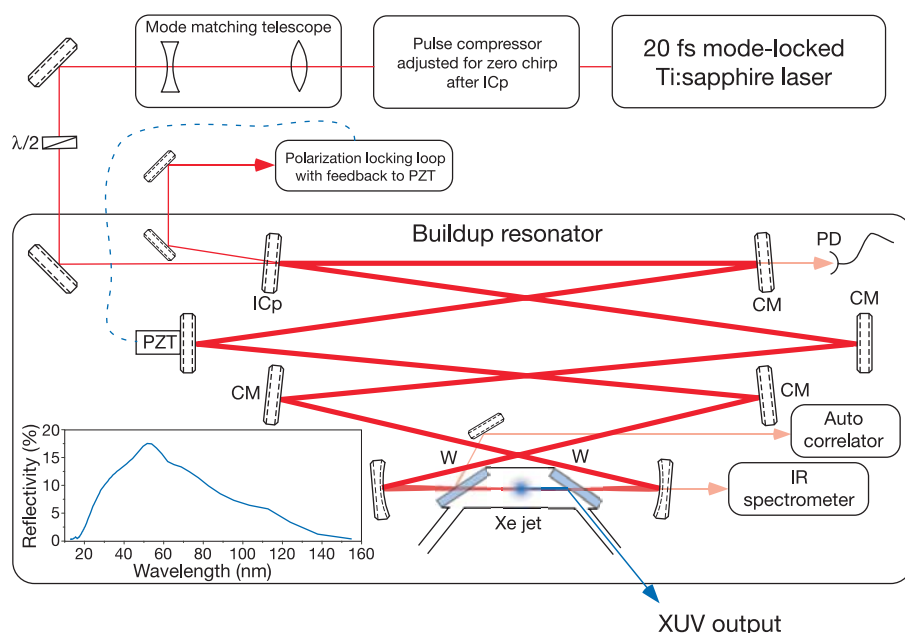


Figure 1 | The XUV laser set-up. ICp, coupling mirror with 1% transmission; CM, chirped mirrors; PD, photodiode; W, Brewster-angled sapphire window of 1 mm thickness; PZT, piezoelectric transducer. Inset shows the reflectance of sapphire for p-polarized XUV light at an incidence angle of 60.4° (Brewster angle for 800 nm radiation).

determination of the optical frequencies. However, applicability of our XUV frequency comb for high resolution laser spectroscopy relies on the coherence of the generation process. So far there was only indirect evidence that this is indeed the case. For example, it was observed that two independent HHG sources pumped by the same fundamental pulse produce stable interference fringes²² and that the generated XUV bursts are compressible²³, which both indicate that the phase link between fundamental and XUV is tight. Direct evidence can only be provided by a beat measurement between two independent XUV sources or a spectroscopy measurement. Upconversion of phase noise in the fundamental pulse train that might bury the comb is reduced in our approach, as the enhancement resonator acts as a flywheel for the incoming pulse train.

When the buildup resonator (shown in Fig. 1 and explained in the Methods section) is put into lock and the CE frequency of the laser is adjusted to maximize the power inside the resonator, an optical spectrum as shown in Fig. 2 is observed. The circulating (average) power has been determined to be 38 W. The discrepancy between the calculated ($P = 100$) and observed enhancement ($P = 54$) can mostly be attributed to spectral filtering due to residual dispersion inside the resonator. The autocorrelation of the stored pulse inside the resonator is measured using the residual reflection of the fundamental laser radiation from the first Brewster window

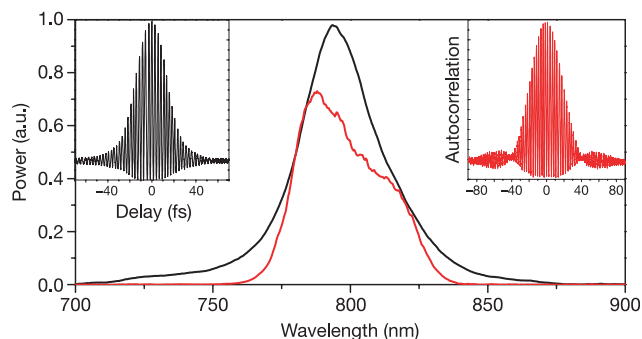


Figure 2 | Normalized spectrum of the pump laser (black) and the circulating pulse in the enhancement resonator (red) when locked. Inset, respective measured autocorrelations.

(see Fig. 1). It indicates an approximately chirp-free pulse of about 28 fs duration. With these figures, we estimate a peak power of about 12 MW and intensities in the focus of around $5 \times 10^{13} \text{ W cm}^{-2}$. Turning on the xenon jet has no effect on the cavity lock, which shows that plasma induced phase noise above feedback bandwidth (10 kHz) is negligible.

Given these conditions, an XUV spectrum as shown in Fig. 3 can be observed after the XUV output coupler, which is described in the Methods section. High harmonics up to fifteenth order or 23 eV photon energy (as expected for these intensities) are observed, with an exponential roll-off starting around the ninth harmonic, presumably due to increasing phase mismatch. The cut-off wavelength of our XUV spectrometer around 120 nm prevented us from observing the fifth harmonic. The third harmonic could be observed with the naked eye, even though the outcoupling efficiency was very low for this wavelength. Additionally, we observe a line slightly below the ionization potential of xenon at 103 nm, which to our knowledge has not been reported previously. To test the comb coherence, we performed a beat experiment between the third harmonic from the

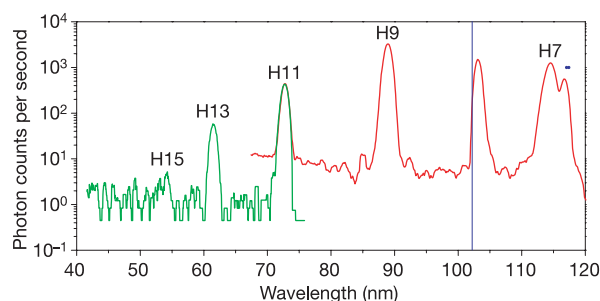


Figure 3 | Harmonic spectrum obtained with the resonator locked and the xenon jet on. The short-wavelength end of the spectrum (green) is taken with a $0.1 \mu\text{m}$ aluminium filter in the beam path to reduce stray light from the stronger lower harmonics, and is rescaled to match the peak height at the eleventh harmonic (H11) of the measurement without filter (red). Blue dots indicate the 5P–7S resonance in xenon that modifies the shape of the seventh harmonic (H7), and the vertical line indicates the ionization potential. The spectral feature just below the ionization limit presumably originates from fluorescence of coherently excited Rydberg states.

enhancement cavity and the fourth harmonic of a mode-locked Nd:YVO₄ laser. The results are shown in Fig. 4. A similar result²⁴ is obtained in a beat measurement between third harmonics produced in a nonlinear crystal and a gas jet, which proves equation (2) for this special case.

In order to test the spatial coherence of the harmonic radiation and investigate the possible origin of the feature at 103 nm, we have recorded transverse beam profiles for each feature of the spectrum from the seventh through to the eleventh harmonic by translating the monochromator in the horizontal direction and recording the transmitted intensity for each feature. A gaussian fit to these profiles yields divergence angles of 14, 11 and 10 mrad for the seventh, ninth and eleventh harmonic, respectively. This corresponds approximately to the diffraction limit, which would allow tight focusing. The divergence of the feature at 103 nm could not be determined, as it exceeds the limited viewing angle of the set-up.

We also investigated the generated power of the different spectral features as a function of the fundamental power. The results are shown in Fig. 5. As expected²⁵, for low intensities before the individual harmonic reaches the plateau, it follows a power law with an exponent close to the harmonic order. As the harmonic reaches the plateau, the exponent decreases to a value that is approximately the same for all harmonic orders. The line at 103 nm exhibits a very high slope for low intensities while it quickly saturates and even decreases at higher powers, which, in addition to the divergence and the frequency not being close to an odd harmonic, suggests that a different process than the usual HHG is responsible for that feature. A possible explanation for this feature would be that during the pulse the Rydberg levels of the atom are Stark-shifted into eight- and even nine-photon resonance with the fundamental laser light. Population can accumulate there, as high-lying Rydberg states are robust against field ionization for such short laser pulses. This long-lived excited dipole can now oscillate for a long time in phase even after the pulse has passed, so that most of the emitted power is directed and has a frequency of the unperturbed Rydberg states just below the ionization limit.

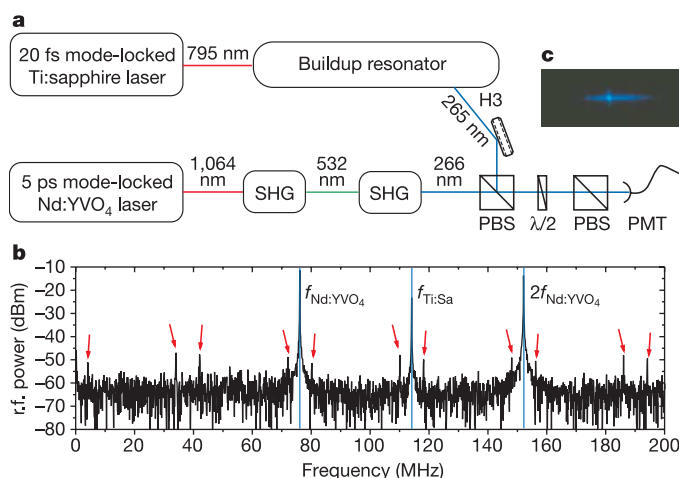


Figure 4 | Frequency comb coherence. **a**, Set-up for testing comb coherence at the third harmonic (H3). SHG, second harmonic generation; PBS, polarizing beam splitter; PMT, photomultiplier. To synchronize the two lasers, the third harmonic of the Nd:YVO₄ laser repetition rate (76 MHz) was phase-locked to the second harmonic of the Ti:sapphire laser repetition rate (changed to 114 MHz). The repetition rates from the two lasers are marked with blue lines in the r.f. spectrum (**b**). The red arrows mark the position of the beat signals observed when the delay between the pulses is adjusted appropriately using an r.f. delay line. **c**, Grating image of the overlapped beams with the narrow bandwidth picosecond laser (bright spot) confined to the centre of the femtosecond spectrum.

The total power of the spectral range from 120 nm down to 60 nm was determined to be more than 1 nW after the sapphire window. If the calculated coupling efficiency of that window is taken into account, more than 10^{-8} of the power from the laser is converted into photons in the 60–120 nm range. Optimized results with chirped pulse amplifier (CPA) systems reached more than 10^{-5} single pass efficiency in this wavelength range²⁶. With the advent of precision dispersion characterization²⁷, dispersion-compensated resonators with enhancement factors of 1,000 are possible, so that with high energy oscillators²⁸, intra-cavity pulse energies approaching 100 μJ seem realistic with current technologies. Obviously, at such high pulse energies, special care has to be taken to avoid nonlinear responses of such a resonator, such as self phase modulation in windows, two-photon absorption in the mirror coatings, and the like. In this case optimized single pass conversion efficiencies would allow a total power conversion in the per cent range and XUV output powers in the mW range.

In conclusion, we have shown that efficient high-order harmonic generation directly from a Ti:sapphire oscillator is possible by use of an enhancement resonator of high finesse specifically designed to support ultrashort femtosecond pulses. This not only reduces the complexity of the set-up for the generation of high harmonics compared to a typical CPA system but simultaneously increases the repetition rate of this conversion by several orders of magnitude. At such a high repetition rate, the frequency comb from the fundamental laser will also be usable in the XUV, so that high resolution spectroscopy in the XUV comes into reach. This will enable new high precision tests for fundamental physical theories. For example, the 1S–2S transition of singly charged helium can be probed with the thirteenth harmonic of a Ti:sapphire laser. This would provide an even more sensitive test of quantum electrodynamics than the hydrogen 1S–2S experiment²⁹. If these measurements are combined, a new precise value for the proton charge radius and the Rydberg constant may be obtained. It can be expected that the presented method is capable of producing orders of magnitude more intense XUV output than conventional HHG schemes and with excellent spatial coherence, so that other applications like XUV interferometry, holography and lithography come into the realm of reality.

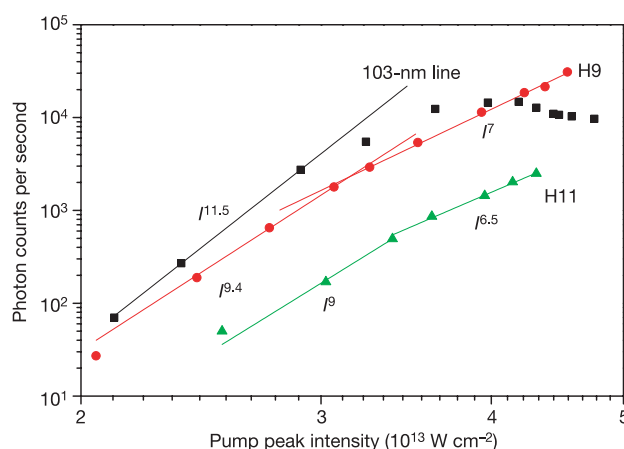


Figure 5 | Generated power versus the fundamental intensity I for three spectral features. Data are shown for the feature at 103 nm (squares), the ninth harmonic (H9; circles) and the eleventh harmonic (H11; triangles). Solid lines represent fits to the data with a power law with exponents as indicated. The ninth harmonic follows a power law with an exponent of 9.4 for low intensities, as expected from lowest order perturbation theory, whereas for higher powers it decreases to 7. The eleventh harmonic shows a similar behaviour, with the change in exponent occurring at a higher intensity. The line at 103 nm shows a high exponent of 11.5 for low intensities and then quickly saturates (and even decreases).

METHODS

Apparatus. The complete set-up is sketched in Fig. 1, and consists of a Ti:sapphire femtosecond oscillator (Femtolasers Femtosource 20) that delivers 20 fs pulses with 850 mW average power at 800 nm and a repetition rate of 112 MHz, which results in a pulse energy of 7.7 nJ and a peak power around 400 kW. These pulses are sent to the enhancement resonator, which is a bowtie-type ring cavity. It consists of eight mirrors: a 1% transmission coupling mirror; a 1/4-inch mirror mounted on a piezoelectric transducer for electronic control of the cavity length using the polarization lock technique³⁰; four plane chirped mirrors for compensation of the dispersion of air; and the two sapphire Brewster windows inside the resonator. Two spherical mirrors with 50 mm focal length produce a tight focus with a diameter of about 5.3 μm inside the harmonic generation chamber. On the way to the resonator, the beam passes through a prism compressor that compensates the dispersion of the coupling mirror substrates and a telescope that matches the beam diameter and focus position to the resonator mode. In this way, nearly transform limited pulses are obtained inside the resonator. For optimal coupling the enhancement resonator input mirror has 1% transmission, so that an enhancement factor of $P = 100$ is expected. The laser resonator includes a pair of thin wedged fused silica plates for manual control of the carrier envelope frequency. No electronic feedback was necessary to control the CE frequency of the laser, because the system was stable enough to operate for hours by feeding back on the resonator length only. The power circulating in the resonator while put into lock was determined by measuring the power leaking through one of the highly reflecting cavity mirrors with accurately known spectral transmission. The intra-cavity spectrum was measured the same way. Amplitude stability was better than 10% r.m.s. with no frequencies higher than 1 MHz limited by photon lifetime.

Procedure. The enhancement resonator contains a small vacuum chamber with Brewster-angled sapphire windows, housing the rare-gas jet. The peak intensity in these windows is smaller than 10 GW cm^{-2} , so that their nonlinear response is negligible. Xenon gas is injected into the focus of the resonator mode in the HHG chamber, using a glass capillary placed right above the focus with approximately 50 μm inner diameter and 1 bar backing pressure. This diameter is approximately matched to the Rayleigh range of the focus of about 110 μm . With this focus geometry and repetition rate, the atoms see about five pulses before leaving the focus. As the coherently generated XUV radiation is emitted collinearly with the fundamental in a well-collimated beam, a crucial component in the scheme is some sort of beam splitter that separates the two. In order not to compromise the resonator finesse, this beam splitter has to have a very low loss for the fundamental laser radiation. The vacuum windows fulfill this requirement through Brewster orientation with losses smaller than 10^{-3} per window, and accomplish the separation by providing total external reflection for the XUV. High Fresnel reflectivity (inset in Fig. 1) is achieved in this range, as the refractive index of sapphire is lower than one for the relevant wavelengths. The generated XUV beam extracted this way was analysed with a grazing incidence XUV monochromator (McPherson model 248/310G) placed at the HHG chamber exit port. A channeltron photodetector (BURLE CEM4751G), sensitive to wavelengths below 150 nm, was installed at the exit slit of the monochromator, and its signal was analysed with (1) a photon counter (Stanford Research SR400) for high sensitivity measurements, and (2) a voltmeter across a 1 M Ω load resistance for XUV photon flux measurements. To determine the total power in the observed XUV spectrum, the channeltron was placed directly in the beam at the XUV port. A photocurrent of 1 μA was detected at 1.36 kV bias, which corresponds to a photon flux of about 0.85×10^9 photons per second, according to the manufacturer's specifications. From the spectrum we know that the major contributions to that flux come from the spectral range between 85 and 115 nm, so that we can assume an average photon energy of about 12 eV, from which the total power can be calculated.

To synchronise the two lasers in the beat experiment, the third harmonic of the Nd:YVO₄ laser repetition rate (76 MHz) was phase-locked to the second harmonic of the Ti:sapphire laser repetition rate (changed to 114 MHz). The delay between the pulses from the two lasers was adjusted using an r.f. phase shifter. The beat signal was recorded using a photomultiplier tube.

Received 15 March; accepted 23 May 2005.

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Acknowledgements We thank A. Apolonski and M. Yu. Ivanov for discussions, and E. Seres and J. Seres for lending us the XUV monochromator.

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LETTERS

Highly controlled acetylene accommodation in a metal-organic microporous material

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Metal-organic microporous materials^{1–4} (MOMs) have attracted wide scientific attention owing to their unusual structure and properties, as well as commercial interest due to their potential applications in storage^{5–9}, separation^{10,11} and heterogeneous catalysis^{12,13}. One of the advantages of MOMs compared to other microporous materials, such as activated carbons, is their ability to exhibit a variety of pore surface properties such as hydrophilicity and chirality, as a result of the controlled incorporation of organic functional groups into the pore walls^{11,13–15}. This capability means that the pore surfaces of MOMs could be designed to adsorb specific molecules; but few design strategies for the adsorption of small molecules have been established so far. Here we report high levels of selective sorption of acetylene molecules as compared to a very similar molecule, carbon dioxide, onto the functionalized surface of a MOM. The acetylene molecules are held at a periodic distance from one another by hydrogen bonding between two non-coordinated oxygen atoms in the nanoscale pore wall of the MOM and the two hydrogen atoms of the acetylene molecule. This permits the stable storage of acetylene at a density 200 times the safe compression limit of free acetylene at room temperature.

Acetylene (C_2H_2) is one of the key molecules as a starting material for many chemical products¹⁶ and electric materials¹⁷ in the petrochemical and electronic industries. To obtain highly pure C_2H_2 for the preparation of these materials, it is important to separate C_2H_2 from the mixture gas containing carbon dioxide (CO_2 ; which is commonly present in many industrial processes¹⁸) impurities without a large expenditure of energy. Zeolites and activated carbons are known to be materials capable of accommodating large amounts of both gases. No relevant difference in capacity has been observed, because these molecules are similar to one another in equilibrium sorption parameters, related physico-chemical properties, and molecular size and shape (Supplementary Fig. S1). Acetylene is well known to be a highly reactive molecule: it cannot be compressed above 0.2 MPa or it explodes without oxygen, even at room temperature¹⁹. It thus appears that potential, safe materials for C_2H_2 separation and storage should be investigated.

Microporous compounds with a cross-section of 4–6 Å are relevant for the accommodation of C_2H_2 and CO_2 molecules: both molecules can be strongly confined in the pore without any chemical interaction, by means of the deep, van der Waals type, potential-energy well²⁰. In this respect, a complex **1**— $Cu_2(pzdc)_2(pyz)$, where $pzdc$ is pyrazine-2,3-dicarboxylate and pyz is pyrazine—is a suitable

adsorbate for the adsorption of these molecules owing to their permanent one-dimensional channels of cross-section of 4 Å × 6 Å (refs 21, 22). As-synthesized complex **1**·2H₂O shows another aspect of the pore. The water molecules as guest molecules are connected to oxygen atoms fixed on the pore surface in a one-to-one fashion, indicating that the oxygen atoms act as basic adsorption sites for guest molecules (Supplementary Fig. S2). Accordingly, we expect that complex **1** would exert an effective sorption ability for small molecules, having acidic parts owing to the deep, van der Waals type, potential-energy well and additional hydrogen-bonding interactions. The C_2H_2 molecule has a linear form with acidic hydrogen atoms at both ends ($pK_a = 25$). The CO_2 molecule has a rod-shaped form with dimensions 3 Å × 5 Å, similar to that of C_2H_2 , but it has no acidic protons. We thus suggest complex **1** as a feasible adsorbate for the C_2H_2 molecule.

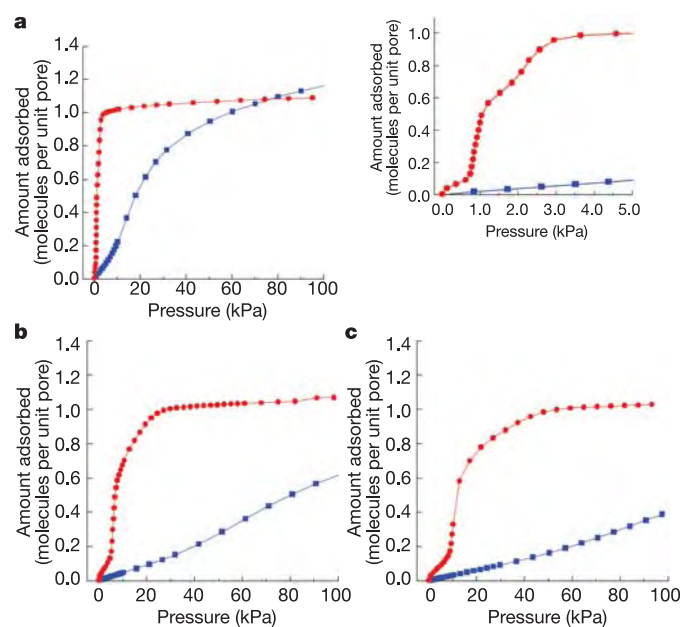


Figure 1 | Adsorption isotherms for C_2H_2 (red circles) and CO_2 (blue squares) on complex **1.** The pressure range is from 10^{-4} to 100 kPa and the temperatures are at 270 K (a; the inset shows the lower P/P_0 region), 300 K (b) and 310 K (c).

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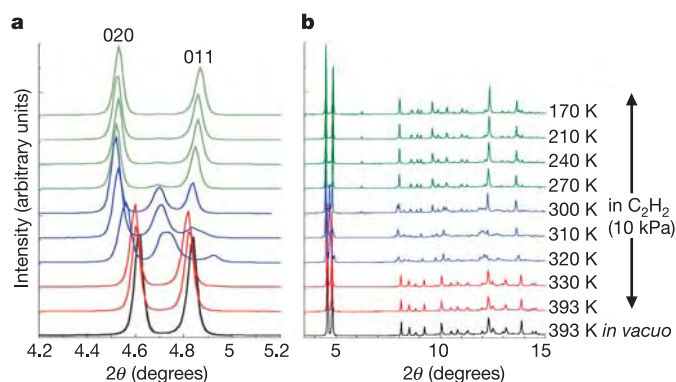


Figure 2 | *In situ* synchrotron XRPD patterns of complex **1** with adsorption of acetylene. The radiation wavelength is 0.80 Å. The 2θ ranges from 4.2° to 5.2° (a) and from 3.5° to 15.0° (b).

The sorption isotherms of C_2H_2 and CO_2 at 270, 300 and 310 K were measured on the anhydrous complex **1** (Fig. 1 and Supplementary Fig. S5). A marked difference in the C_2H_2 and CO_2 adsorption isotherms was observed. The adsorption isotherms of C_2H_2 show a steep rise in the very-low-pressure region and reach saturation, whereas those of CO_2 indicate gradual adsorption. The saturation-adsorbed amount of C_2H_2 , $42 \text{ cm}^3 (\text{at STP}) \text{ g}^{-1}$, corresponds to just one molecule per unit pore. The maximum ratio of amount of C_2H_2 adsorbed to amount of CO_2 adsorbed is 26.0 (at 1.1 kPa at 270 K), indicating that complex **1** adsorbed C_2H_2 more preferentially than CO_2 at the low pressure and the ambient temperature, which would provide a safe, energy-saving separation process. We calculated the isosteric heats (q_{st}) of C_2H_2 and CO_2 by using the Clausius–Clapeyron equation. The obtained q_{st} value at the fractional filling of 0.2 of C_2H_2 , 42.5 kJ mol^{-1} , is higher than that of CO_2 , 31.9 kJ mol^{-1} . These results indicate that C_2H_2 and complex **1** interact better than do CO_2 and complex **1**.

To elucidate the mechanism of high adsorption ability for C_2H_2 molecules in complex **1**, we determined the structure of complex **1** with adsorbed C_2H_2 molecules by the MEM (maximum entropy method)/Rietveld analysis using synchrotron X-ray powder diffraction data (XRPD)^{21,23,24}. Figure 2 shows *in situ* XRPD patterns of anhydrous complex **1** with C_2H_2 at 10 kPa with cooling from 393 K to

170 K. During the cooling process, a new diffraction peak at 4.7° (lattice spacing $d = 9.7 \text{ Å}$) in 2θ appeared at 320 K, which could not be indexed either with the same unit cell or with a combination of cells with the apohost (complex **1** without the guest molecule adsorbed). Therefore, the new diffraction peak in the range $270 \text{ K} < T < 330 \text{ K}$ is due to another phase in the dynamic crystal transformation. The intensity of this diffraction peak weakened and another new diffraction peak at $\sim 4.9^\circ$ in 2θ appeared, the intensity of which increased with further cooling. Finally, an almost single phase appeared at 270 K (see also Supplementary Discussion and Fig. S4).

The overall crystal structure and electron density of complex **1** with C_2H_2 at 10 kPa at 170 K were obtained by the MEM/Rietveld analysis (Fig. 3 and Supplementary Fig. S3). Only one C_2H_2 molecule locates in the middle of the channels. The stoichiometry of one C_2H_2 molecule per unit pore is in good agreement with that of the adsorption measurement. The micropore volume of the unit pore is 99.7 Å^3 , which is calculated as the molecular accessible volume of the crystal structure, and so the density of adsorbed C_2H_2 is estimated to be 0.434 g cm^{-3} ; this value of density is equivalent to the value of an imaginary state of acetylene at 41 MPa at room temperature and is 200 times larger than the value of the compression limit for the safe use of C_2H_2 at room temperature¹⁹, $0.20 \text{ MPa} = 0.0021 \text{ g cm}^{-3}$.

In the channel, the C_2H_2 molecules align along the a axis with an inclination of 78.1° , and the intermolecular distance is 4.8 Å , indicating that they are densely packed with a short intermolecular distance while avoiding the close contact that induces an explosion. Each end of the C_2H_2 molecule is oriented to the two non-coordinated oxygen atoms on the pore wall. The distance between one hydrogen atom of C_2H_2 and the oxygen atom is 2.2 Å , which is smaller than the sum of the van der Waals radius of hydrogen and oxygen atoms, 2.6 Å , indicating that O—H—C hydrogen bonds exist. We carefully inspected the electron density distribution on the two-dimensional section along the molecular axis of the C_2H_2 molecule by the MEM analysis (Fig. 4). As a result, we can recognize a few electrons ($0.21 \text{ electrons Å}^{-3}$) between the hydrogen atom of C_2H_2 and the free oxygen atom, indicating that this interaction is based not only on electrostatic attraction but also on the electron delocalization effect between H and O atoms. These interactions strongly fix the C_2H_2 molecule in a periodic unit pore and isolate the C_2H_2 in the one-dimensional channel, which gives rise to the enhancement of the ‘confinement effect’ and enables the stable accommodation of C_2H_2

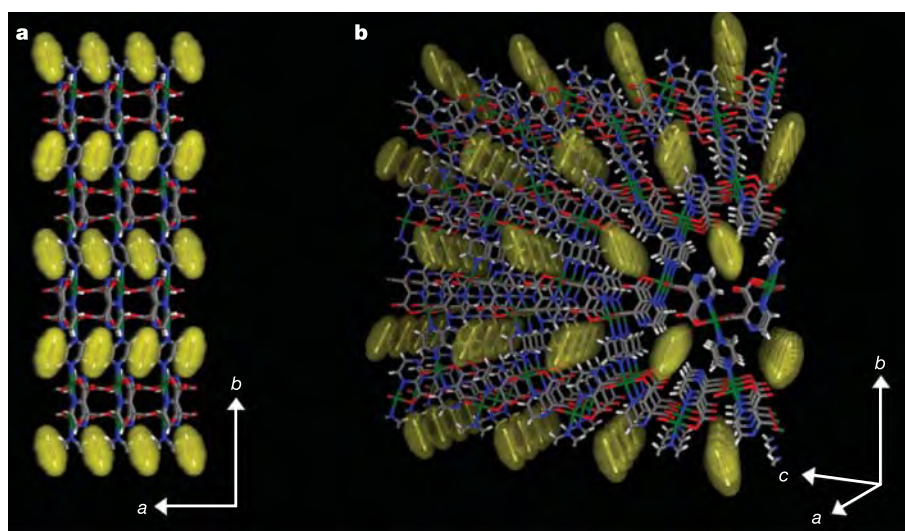


Figure 3 | Crystal structure of complex **1** with C_2H_2 at 170 K from MEM/Rietveld analysis. Cu, green; O, red; C, grey; N, blue; H, white. **a**, Orthographic views down the c axis. **b**, Perspective views down the a axis.

Crystal data are as follows: $\text{C}_9\text{H}_5\text{CuN}_3\text{O}_4$, $M = 282.70$, monoclinic, space group $P2_1/c$ (no. 14), $a = 4.81352(4) \text{ Å}$, $b = 20.23550(15) \text{ Å}$, $c = 10.71646(11) \text{ Å}$, $\beta = 96.9366(11)^\circ$, $V = 1036.18(3) \text{ Å}^3$, $Z = 4$.

molecules (see also Supplementary Discussion and Supplementary Figs S6 and S7).

In situ Raman spectra of complex **1** with C₂H₂ (10 kPa) and without guest molecule during cooling from 300 K to 170 K show a strong band at 1,946.5 cm⁻¹ assigned to the stretching mode of C≡C, which has a slightly lower frequency than that of bulk acetylene crystal at 173 K and 1,956 cm⁻¹, and at 77 K and 1,948 cm⁻¹ (ref. 25; see also Supplementary Methods and Supplementary Fig. S12). This slight shift could be due to the combination of double hydrogen bonding and nanosized confinement effects²¹.

The results of first-principles calculations using ultrasoft pseudopotentials and the generalized gradient approximation²⁶ also indicate the specific stabilization of the acetylene adsorption system. The methods used permit accurate adsorption energies to be calculated from the reactant–product energy differences, between the binding energy of the adsorbed system and those of empty complex **1** and free C₂H₂ molecules (Supplementary Fig. S8). The energy balance of complex **1** is expressed by the following equation:

$$E_{\text{host+guest}} = E_{\text{apohost}} + E_{\text{guest}} + \Delta E_{\text{ads}} \\ = E_{\text{host}} - \Delta E_{\text{trans}} + E_{\text{guest}} + \Delta E_{\text{ads}}$$

where the calculated binding energies of the anhydrous structure before adsorption (E_{apohost}) and the acetylene adsorbed structure ($E_{\text{host+guest}}$) are estimated to be $E_{\text{apohost}} = -283.885$ eV and $E_{\text{host+guest}} = -307.400$ eV, respectively. In addition, the guest-only (E_{guest}) and host-only (E_{host}) contributions to the binding energy of the acetylene adsorbed structure are estimated to be $E_{\text{guest}} = -23.110$ eV and $E_{\text{host}} = -283.815$ eV, respectively. The

structural transformation energy, $\Delta E_{\text{trans}} = 6.8$ kJ mol⁻¹, indicates that this structural transformation is energetically unfavourable for the host framework. However, the calculated heat of adsorption, ΔE_{ads} , shows a large negative value, -39 kJ mol⁻¹, indicating that the porous compound gets enough energy return by the adsorption of acetylene molecules, which compensate the energy loss of the structural transformation.

The specific sorption ability of complex **1** for C₂H₂ is ascribed to the binding of the acetylene molecule by the two basic oxygen atom sites, which are ideally arranged on the regular micropore surface. Usually, small molecules such as CO₂ are adsorbed in the micropore only by the deep, van der Waals type, potential-energy well. However, these functional sites of flexible MOMs attain optimal chemical bonding with specific adsorbates and significantly enhance the 'confinement effect'. Eventually, stoichiometric guest incarceration is observed, with commensurate structures between guest array and host. The results show that when the multiple specific interaction sites are located at suitable positions on the regular micropore, an adsorption system specific to the target molecule can be realized. This new guideline for specific adsorption systems on microporous materials will be applicable to a wide range of target molecules, because MOMs having various pore sizes and functional surface properties can be synthesized.

METHODS

Synthesis of Cu₂(pzdc)₂(pyz)·2H₂O (hydrated complex **1).** A mixture of Na₂pzdc (0.21 g, 1.00 mmol) and pyz (1.0 g, 12.5 mmol) dissolved in H₂O/EtOH (1:1, 100 ml) was slowly added to the H₂O solution (100 ml) containing Cu(ClO₄)₂·6H₂O (0.37 g, 1.00 mmol) at room temperature. After stirring for one day, the resultant precipitate was filtered, washed with H₂O and methanol, and dried under reduced pressure²⁷. Elemental analysis for C₁₆H₁₂Cu₂N₆O₁₀ is as follows. Calculated: C, 33.40; H, 2.10; N, 14.61. Found: C, 32.78; H, 1.57; N, 14.34.

Adsorption measurements. The adsorption isotherms of acetylene and carbon dioxide were measured with BELSORP18 volumetric adsorption equipment from Bel Japan. Acetylene gas and carbon dioxide of high purity (99.99% and 99.9999%, respectively) were used. The host sample was obtained by treating under reduced pressure (<10⁻² Pa) at 398 K for more than 10 h. Each point in the adsorption isotherms has an error of ±0.25%, which is caused by the resolution of the pressure gauge (see also Supplementary Fig. S11).

X-ray powder diffraction experiments. The powder samples for each compound were sealed in a silica glass capillary (0.4 mm inside diameter). The XRPD pattern with good counting statistics was measured by the synchrotron radiation XRPD experiment with the large Debye–Scherrer camera and imaging plate as detectors on the BL02B2 beam line at the Super Photon Ring (SPring-8, Hyogo, Japan)²⁸. All the XRPD patterns were obtained with a 0.01° step (see also Supplementary Methods).

Structure determinations by MEM/Rietveld analysis. The structure determinations were performed using high-brilliance synchrotron powder diffraction data for complex **1** with C₂H₂ at 10 kPa and 170 K is determined with the same space group, *P2₁/c*, as complex **1** without the guest molecule. The values of R_{wp} and R_1 as reliability factors of Rietveld fitting are 1.74 and 3.20%, respectively. The final MEM electron density, with reliability factor $R_{\text{MEM}} = 1.76\%$, clearly revealed that ellipsoidal electron densities exist in the centre of the channels, which can be assigned by the electron densities of one C₂H₂ molecule (see also Supplementary Data, Supplementary Methods, Supplementary Table S1 and Supplementary Figs S9 and S10).

Theoretical calculations. The first-principles calculations were performed with density functional theory, using the pseudopotential method and a plane-wave basis set. Exchange correlation was included using the Perdew–Wang (PW91) generalized gradient-corrected functional given by ref. 26. The energy cut-off was set to 400 eV and the convergence in energy and force were 10⁻⁴ eV and 3 × 10⁻³ eV Å⁻¹, respectively. The (8 × 2 × 4) Monkhost–Pack k-point mesh was used²⁹. All calculations were performed using the Venna *ab initio* Simulation Package³⁰.

Received 6 January; accepted 12 May 2005.

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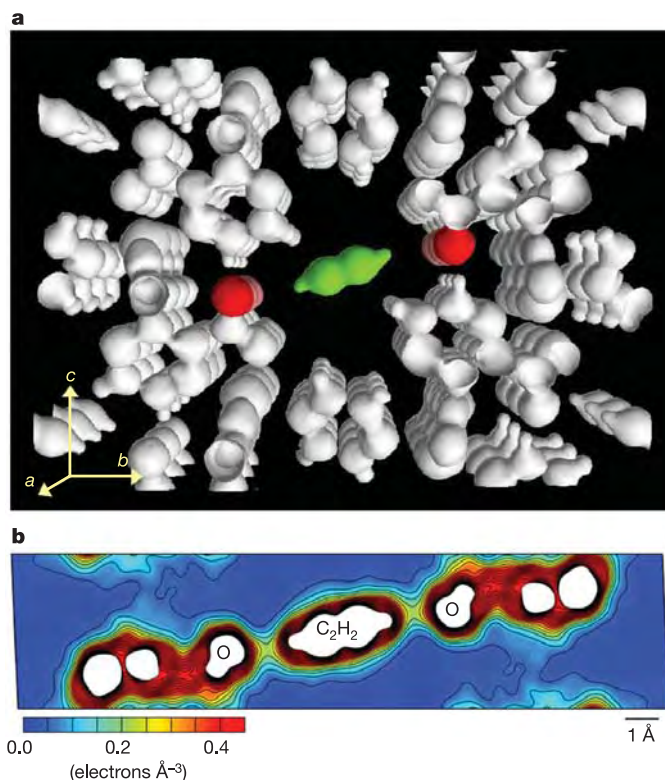


Figure 4 | MEM electron density views. **a**, MEM electron densities of C₂H₂ adsorbed in **1** at 170 K as an equal-density contour surface along the *a* axis. The equicontour level is 1.0 electrons Å⁻³. The central C₂H₂ molecule and oxygen atoms are represented by yellow-green and red, respectively. The other atoms are represented by white. **b**, MEM electron density distribution views on the two-dimensional section defined by the molecular axis of the C₂H₂ molecule and the *a* axis.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements We thank H. Tanaka, K. Kato and the staff of the Center for Computational Materials Science at the Institute for Materials Research, Tohoku University. The synchrotron radiation experiments were performed at the BL02B2 in SPring-8. This work was supported by a Grant-In-Aid for Science Research in a Priority Area 'Chemistry of Coordination Space' from the Ministry of Education, Science, Sports and Culture, Japan.

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LETTERS

Regional insolation forcing of late Quaternary climate change in the Southern Hemisphere

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In agreement with the Milankovitch orbital forcing hypothesis¹ it is often assumed that glacial–interglacial climate transitions occurred synchronously in the Northern and Southern hemispheres of the Earth. It is difficult to test this assumption, because of the paucity of long, continuous climate records from the Southern Hemisphere that have not been dated by tuning them to the presumed Northern Hemisphere signals². Here we present an independently dated terrestrial pollen record from a peat bog on South Island, New Zealand, to investigate global and local factors in Southern Hemisphere climate changes during the last two glacial–interglacial cycles. Our record largely corroborates the Milankovitch model of orbital forcing but also exhibits some differences: in particular, an earlier onset and longer duration of the Last Glacial Maximum. Our results suggest that Southern Hemisphere insolation may have been responsible for these differences in timing. Our findings question the validity of applying orbital tuning to Southern Hemisphere records and suggest an alternative mechanism to the bipolar seesaw for generating interhemispheric asynchrony in climate change.

Local insolation variation in the mid-latitudes of the Southern Hemisphere is almost completely out of phase with insolation variation at the high northern latitudes, so it is plausible that global signals of climate change are modulated or even driven by local insolation effects in the south. For example, it has long been argued, but not established³, that the Southern Hemisphere may have led the Northern Hemisphere into (and out of) glaciations⁴. This assertion implies a southern ‘trigger’, perhaps involving local insolation forcing, amplified in some way by the Antarctic ice sheet and its influence on global sea level, ocean circulation or atmospheric circulation in the form of the circum-Antarctic westerly winds⁵.

Southern New Zealand is a prime location for examining interhemispheric linkages in climate change. It is located in the mid-latitudes of the Southern Ocean directly in the path of the southern westerly winds, and has high mountains (the Southern Alps) that support glaciers that are sensitive to short-term climate change. The deposits and landforms of these glaciers provide a complex record of late Quaternary ice advance⁶, which has been linked to global climate events⁷. Associated sedimentary sequences provide opportunities for comparing terrestrial changes in southern New Zealand with marine and Antarctic records. Okarito Pakihi (43° 14′ 30″ S, 170° 13′ E, 70 metres above sea level, m.a.s.l.) is a moraine-impounded peat bog over 10 m deep (Supplementary Fig. 1) situated outside the limits of the Last Glacial Maximum (LGM) ice advance on the West Coast of South Island, New Zealand. Lowland podocarp/hardwood forest dominated by *Dacrydium cupressinum* surrounds the site, montane and subalpine low forest and shrubland occurs regionally

between 400 and 1,200 m.a.s.l. and alpine grassland above 1,200 m.a.s.l. (ref. 8). The pollen record at Okarito Pakihi shows temporal shifts in these regional altitudinal vegetation zones, which today are mainly controlled by temperature⁸.

Multiple cores, recovered using a 5-cm-diameter square-rod piston corer and Russian D-section corer, show consistent stratigraphy across the bog (Supplementary Fig. 1). This comprises two dark-brown organic units and two pale-blue-grey micaceous silty sand units, the lower of which is laminated (Fig. 1). Two cores (OKA1 and 913) that represent the entire sequence were sampled for pollen (Fig. 1). An age scale for the record has been established through radiometric dating and tephrochronology from the core OKA1 and replicate cores from the site (Supplementary Information). Radiocarbon (¹⁴C) dating (reported here as calibrated kyr, 1 kyr = 1,000 yr) on bulk sediment, wood and pollen concentrates provide the chronology back to ~22 kyr ago (ref. 9) with the Kawakawa Tephra giving an age tie point at ~26.5 kyr ago (ref. 10). Luminescence dating of the upper micaceous silts provide ages from ~12.5 kyr (pooled average, *n* = 4) below the upper organic bed to 48–75 kyr for the lower part of the unit. A pooled weighted mean age of 127 ± 29 kyr was obtained for the basal laminated silts (Fig. 1, Supplementary Fig. 2, Supplementary Tables 2 and 3). The lower organic unit (Fig. 1) contains no detectable ¹⁴C and is also less suitable for luminescence dating (Supplementary Information). These age constraints suggest that the lower organic unit is older than ~48 kyr (most of Marine Isotope Stage (MIS) 3), and was presumably deposited during the Last Interglacial (MIS 5, 125–80 kyr; ref. 11). The lowermost laminated silty sand unit is therefore likely to have been deposited during the latter stages of the penultimate glaciation (MIS 6). These assertions underpin the following correlation of the Okarito pollen record with the marine isotopic record.

The presence of aquatic taxa, in particular *Isoetes*, within silty sediments, indicates that the basin persisted as a lake from MIS 6 until the end of MIS 2. Pollen of alpine grassland and subalpine shrub communities in the lower laminated inorganic silts indicates cold moist conditions and substantial lowering of the treeline during MIS 6. The combination of inorganic laminated basal sediments in a moraine-bounded basin and cold-climate pollen assemblages indicates close proximity of a glacier at this time. Two prominent grass peaks reflect periods of pronounced cooling and suggest that at least two ice advances occurred during MIS 6. High levels of beech pollen could be due to long-distance transport across an open landscape but may also indicate that during MIS 6 beech forest extended beyond its contemporary geographic range in south Westland.

The dominance of pollen from podocarp/hardwood forest, small trees and shrubs in the overlying organic silt unit indicates major

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forest expansion during MIS 5. Peaks in the podocarp/hardwood taxa indicate three periods of forest expansion, separated by two intervals when montane–subalpine shrubland became more prominent (Fig. 1). Although there is no direct age control for this interval in the record, this pattern is consistent with the fivefold subdivision of MIS 5 with periods of podocarp/hardwood forest expansion, presumably corresponding to substages 5e, 5c and 5a.

Subalpine taxa dominate the upper silt unit, dated to MIS 4–2, with grassland taxa becoming more abundant upwards. We identify six events of expanded grassland communities during this interval, indicating substantial lowering of the treeline, which agrees well with the glacial geomorphic record of the Last (Otira) Glaciation from north Westland¹² and with interpretations of ice advance derived from independent loess and speleothem records from western New Zealand^{6,13}. Luminescence dating (Fig. 1, and Supplementary Information) suggests that the lowermost major grass peak probably represents the MIS 4 (Loopline) ice advance¹². The Kawakawa Tephra (~26.5 kyr) is straddled by the two most prominent grass-pollen peaks, which occur during late MIS 3 and MIS 2 and probably represent the Larrikins (a1 and a2) ice advances¹².

Expansion of montane–subalpine shrubland occurred at around 17.3 kyr ago—at the same time as Westland glaciers underwent massive retreat from their glacial maximum limits¹⁴. Similar marked

climate amelioration is indicated in northern New Zealand at this time in a variety of climate proxies¹⁵. At Okarito, this amelioration eventually culminated in the development of lowland podocarp/hardwood forest at around 11 kyr ago. A possible minor reversal in this forest development phase between ~14.4 and 11 kyr ago, encompassing both the Antarctic Cold Reversal and Younger Dryas chron, does not appear to have been as significant in other West Coast sites¹⁶ but is evident elsewhere in New Zealand^{17,18}.

The independently dated pollen record from Okarito Pakihi is very similar to oxygen-isotope, carbonate and pollen records from DSDP (Deep Sea Drilling Project) Site 594 (Fig. 2), showing that deposition of terrigenous silts off the east coast of the South Island coincided with glacial advance and treeline lowering to the west of the Southern Alps. This is unequivocal evidence for a closely coupled ocean–atmosphere–terrestrial system in the Southwest Pacific during the last two glacial–interglacial cycles, as has been demonstrated for the Southeast Pacific at similar latitudes for the past 50 kyr (ref. 19). Figure 2 also compares our record with an oxygen isotope record from the Indian Ocean, MD 90-0963, thought to be driven principally by orbital forcing at high Northern Hemisphere latitudes, and with the temperature record derived from the Vostok ice core. The climate trends at Okarito are broadly consistent with the northern driver model (Fig. 2).

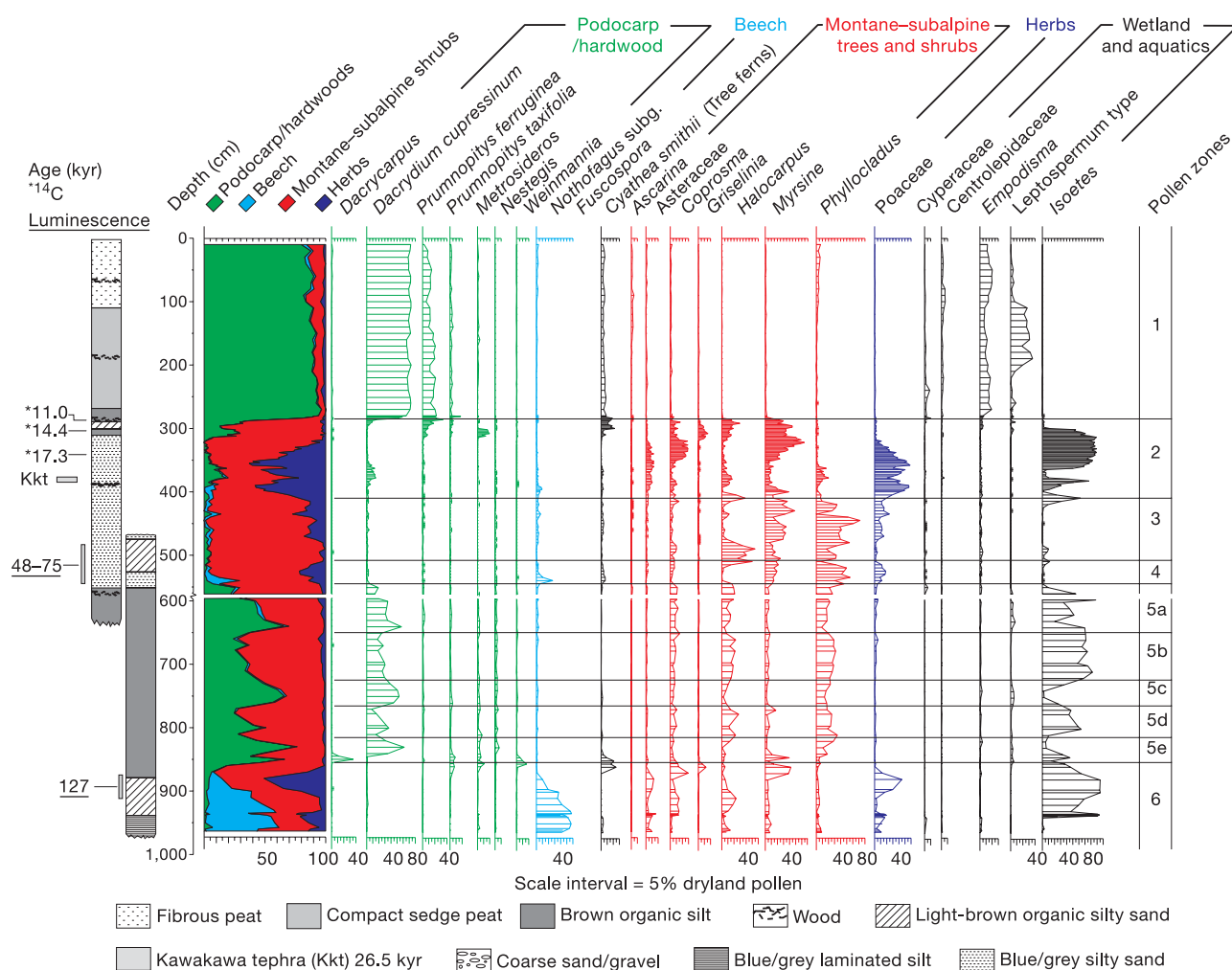


Figure 1 | Condensed pollen diagram from Okarito Pakihi. Luminescence ages (underlined) and radiocarbon ages (asterisked) represent the most reliable ages for the stratigraphy (Supplementary Information). Radiocarbon ages represent a pooled weighted mean of the age results indicated by Ψ in Supplementary Table 1 at 2σ level in calibrated kyr.

Luminescence ages are correlated from replicate cores (see Supplementary Information). A pooled weighted mean age of 127 ± 29 kyr is derived from IRSL (infrared-stimulated luminescence) ages from core 0112b (Supplementary Table 2). Core sediment stratigraphy is explained by the key at base of figure.

There is however at least one significant difference. The onset of maximum cooling during the last glaciation (MIS 4–2) occurs well before the MIS 2 boundary (25–24 kyr ago) as defined in the marine isotopic record¹¹ and hence well before the summer insolation minimum in high northern latitudes. Although not precisely dated at Okarito, the first of the prominent MIS 3/2 grass-pollen peaks begins significantly below the 26.5-kyr ago Kawakawa Tephra, perhaps by as much as 2–4 kyr, at about 28.5–30.5 kyr ago (Fig. 1). An early LGM onset is also evident in speleothem, pollen and glacial geomorphic records from southern New Zealand and Chile^{20,21,22}, and in sea surface temperature reconstructions^{19,23} and isotopic, pollen and carbonate records from offshore marine locations (Fig. 2). In particular, we note the strong correspondence between the grass-pollen curve at Okarito and Taiquemó in southern Chile. The Chilean record shows that an equivalent early LGM onset occurred at about 30 kyr ago (Fig. 2), and hence this pattern may be characteristic of the mid-southern latitudes. It is also notable that the Vostok temperature record begins a marked decline towards

glacial maximum values before 30 kyr (Fig. 2). Although our chronology is at present too imprecise for robust comparison at the millennial scale, an apparent strong correspondence through the Okarito pollen record with the temperature record from Vostok points to consistent teleconnections between the southern mid-latitudes and Antarctica.

The evidence for early onset of maximum glaciation provides renewed support for a Southern Hemisphere ‘lead’ into the LGM and some indication of its cause. Strong cooling in the south commences during, or soon after, the phase when perihelion occurs during the Austral winter (30–35 kyr ago), which means that the local insolation budget was at its lowest level for the entire precessional cycle (Fig. 2). At the same time, insolation in the Northern Hemisphere was still in a positive phase, giving a local radiation budget higher than that at present. The northern ‘driver’ is therefore an unlikely trigger for the onset of maximum glaciation in the south and cannot have been directly responsible for a Southern Hemisphere lead. We propose instead that the early onset of the LGM in the Southern Hemisphere was driven by the minimum in regional insolation reached at 35–30 kyr ago and propagated across the Southern Ocean by geophysical phenomena related to the Antarctic ice sheets, in particular, meridional displacement of sea ice, the circumpolar current system and related westerly winds. The subsequent progressive downturn in northern insolation, however, culminating in the Northern Hemisphere glacial maximum at about 25–24 kyr ago, appears to have superseded local insolation (now in a positive phase) as the driving factor in maintaining cold conditions and glaciation in both hemispheres for the remainder of the LGM. The subsequent maximum in southern insolation around 20 kyr ago may then have promoted the early deglacial warming long recognized in Southern Hemisphere records (for example, ref. 4).

If local insolation was responsible for a Southern Hemisphere lead into the LGM, then similar departures from the orbital forcing model may occur at other times of Southern Hemisphere insolation minima. Chronological control in older parts of the Okarito record does not currently permit a full examination of this postulated mechanism at earlier times. We nevertheless note that the Okarito pollen record and DSDP 594 do not show a strong thermal maximum for the Last Interglacial (MIS 5e) and speculate that this unexpected result may also be linked to strongly reduced local insolation at that time (Fig. 2). Other southern mid-latitude records, however, do not show a similar muted MIS 5e signal^{23,24}.

Recent efforts to explain interhemispheric patterns of Quaternary climate have concentrated on abrupt change at or below the millennial scale with particular attention given to comparison between Greenland and Antarctic ice core records. Much of this work suggests that changes in formation of North Atlantic Deep Water have triggered an asynchronous seesaw response in at least the high latitudes of the Southern Hemisphere. Testing this model against natural archives, however, is predicated on the assumption that millennial-scale patterns are superimposed on interhemispheric synchrony at the Milankovitch scale. Our record of mid-latitude climate in the Southern Hemisphere, while generally conforming to the Milankovitch model, suggests that regional insolation variation may play a greater role in climate change than has previously been attributed and additionally may provide a plausible alternative to the bipolar seesaw to explain differences in interhemispheric climate records.

Received 20 August 2004; accepted 13 May 2005.

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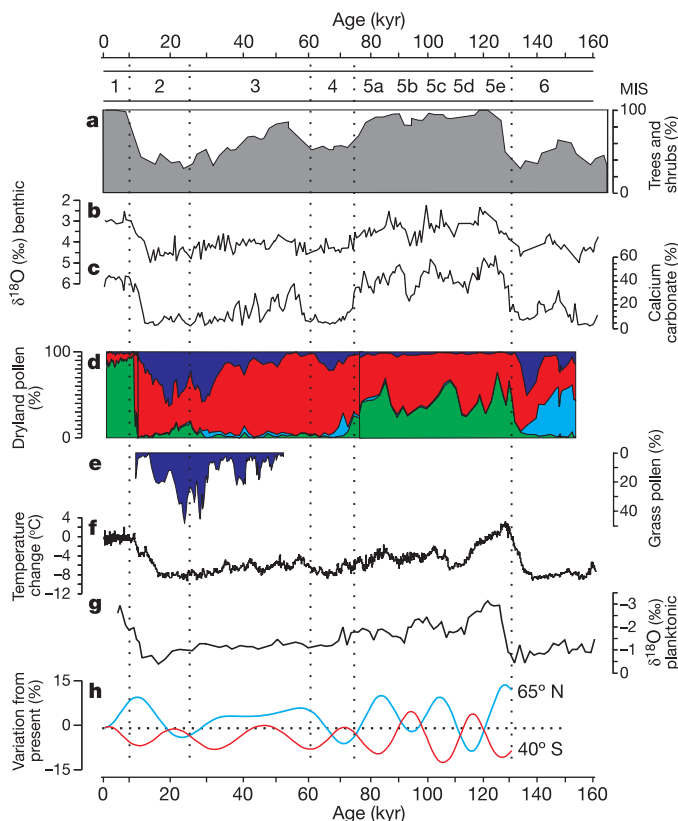


Figure 2 | Comparison of Okarito pollen and other records of climate change with summer insolation variation curves for the past 160,000 yr. **a–c**, DSDP 594 pollen, benthic $\delta^{18}\text{O}$ and calcium carbonate records^{25,26}; **d**, Okarito Pakihi pollen stratigraphy (see Fig. 1 for shading key); **e**, Taiquemó (HE94-2B) grass pollen curve for the past 50 kyr (ref. 21); **f**, Vostok temperature reconstruction²⁷; **g**, MD 90-0963 planktonic $\delta^{18}\text{O}$ record²⁸; **h**, Plot of summer insolation for 40° S and 65° N (ref. 29; data is archived at the World Data Center for Paleoclimatology, Boulder, Colorado, USA. <http://www.ncdc.noaa.gov/paleo/forcing.html>). The climate trends at Okarito show broad similarities with records that invoke a northern driver including maximum glaciation before 130 kyr and from 25–10 kyr with less pronounced cooling from 60–75 kyr; a fivefold subdivision between 75 and 130 kyr; subdued warming from 26–60 kyr relative to the Holocene and much of the period between 75–130 kyr; and broadly synchronous warming at around 12–11 kyr. An early onset of LGM cooling at approximately 28.5–30.5 kyr, however, precedes the northern insolation minimum at about 24 kyr but occurs soon after the southern insolation minimum at 32 kyr.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements We thank M. McGlone, P. Almond, P. Suggate, N. Roberts and D. Charman for discussion on the manuscript. We thank M.-F. Loutre for providing the insolation values used in Fig. 2 and P. Almond for tephra identification. We acknowledge the support of A. Hilgers and U. Radtke and the luminescence dating facilities of the Geographisches Institut, Universität zu Köln, Germany, and the South Westland Department of Conservation, New Zealand. This research is supported by the Natural Environmental Research Council, the Royal Society of New Zealand Marsden Fund, the Royal Society (UK), the Swiss Nationalfonds, and the University of Otago Research Grants Committee.

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LETTERS

Trace-element fractionation in Hadean mantle generated by melt segregation from a magma ocean

Guillaume Caro¹, Bernard Bourdon¹, Bernard J. Wood² & Alexandre Corgne³

Calculations of the energetics of terrestrial accretion indicate that the Earth was extensively molten in its early history¹. Examination of early Archaean rocks from West Greenland (3.6–3.8 Gyr old) using short-lived ¹⁴⁶Sm–¹⁴²Nd chronometry indicates that an episode of mantle differentiation took place close to the end of accretion (4.46 ± 0.11 Gyr ago)^{2–4}. This has produced a chemically depleted mantle with an Sm/Nd ratio higher than the chondritic value. In contrast, application of ¹⁷⁶Lu–¹⁷⁶Hf systematics to 3.6–3.8-Gyr-old zircons from West Greenland indicates derivation from a mantle source with a chondritic Lu/Hf ratio^{5–7}. Although an early Sm/Nd fractionation could be explained by basaltic crust formation⁸, magma ocean crystallization² or formation of continental crust, the absence of coeval Lu/Hf fractionation is in sharp contrast with the well-known covariant behaviour of Sm/Nd and Lu/Hf ratios in crustal formation processes⁵. Here we show using mineral–melt partitioning data for high-pressure mantle minerals that the observed Nd and Hf signatures could have been produced by segregation of melt from a crystallizing magma ocean at upper-mantle pressures early in Earth's history. This residual melt would have risen buoyantly and ultimately formed the earliest terrestrial protocrust.

Early Archaean rocks from West Greenland are characterized by ¹⁴²Nd/¹⁴⁴Nd ratios more radiogenic than those expected for the bulk silicate Earth³ (BSE). These isotopic anomalies result from the decay of the short-lived radionuclide ¹⁴⁶Sm (half-life, 103 Myr) in a mantle characterized by an elevated Sm/Nd ratio. A model age of mantle differentiation can then be estimated by coupling ¹⁴⁷Sm–¹⁴³Nd and ¹⁴⁶Sm–¹⁴²Nd systems in the context of a simple two-stage differentiation model^{2–4}. When this model is applied to Isua metasediments, the timing of the differentiation of the mantle source of these rocks is estimated to be near the final stage of terrestrial accretion, at 4.46 ± 0.11 (2 s.d.) Gyr ago². As Sm is usually more compatible than Nd in mantle minerals, this observation can be explained either by melting of a mantle mineral assemblage or by crystallization of mantle minerals starting from a molten state. Whereas partition coefficients for relatively low-pressure mantle minerals have been available since the 1990s, more complete data sets of partition coefficients for lower-mantle minerals have only been obtained recently^{9–11} and now make it possible to more accurately test scenarios involving crystallization of a magma ocean.

The trace-element fractionations recorded by ¹⁴⁶Sm–¹⁴²Nd and ¹⁷⁶Lu–¹⁷⁶Hf isotopic systems in early Archaean rocks from Amitsoq (West Greenland) are significantly different from those observed in present-day depleted mantle. While an early increase in mantle Sm/Nd ratio is indicated by ¹⁴²Nd/¹⁴⁴Nd ratios more radiogenic than BSE³, 3.6–3.8-Gyr-old zircons from Amitsoq have initial ¹⁷⁶Hf/¹⁷⁷Hf ratios^{5,6} indistinguishable from BSE, requiring derivation of these rocks from a mantle source with a chondritic ¹⁷⁶Lu/¹⁷⁷Hf ratio (see Supplementary Information). These observations are seemingly at

odds, because Lu/Hf and Sm/Nd fractionations are generally coupled during crustal formation processes⁵. Bizzarro *et al.*¹² have argued that these conflicting results may arise from the use of an underestimated ¹⁷⁶Lu decay constant ($\lambda^{176}\text{Lu}$). Indeed, the $\lambda^{176}\text{Lu}$ value estimated by these authors on the basis of meteorite age comparison ($(1.983 \pm 0.033) \times 10^{-11} \text{ yr}^{-1}$) is $\sim 6\%$ higher than estimates made by calibration against the U–Pb decay scheme in terrestrial minerals ($(1.865 \pm 0.015) \times 10^{-11} \text{ yr}^{-1}$)⁷; errors are 2 s.d. Using the former estimate would imply non-chondritic Hf isotope systematics for Amitsoq¹², in line with ^{146,147}Sm–^{142,143}Nd results³. This, in turn, would indicate coupled Lu/Hf and Sm/Nd fractionations consistent with extraction of basaltic or continental crust early in Earth's history. However, ¹⁷⁶Lu–¹⁷⁶Hf systematics of meteorites could be potentially biased by a number of processes^{13,14}, and the consistency of $\lambda^{176}\text{Lu}$ determinations obtained by studies of terrestrial minerals^{7,14} with a wide range of ages strongly suggests that the $\lambda^{176}\text{Lu}$ value initially obtained by Scherer *et al.*⁷ is reliable and applicable to terrestrial samples. Another potential source of discrepancy arises from uncertainties in the ¹⁷⁶Lu–¹⁷⁶Hf chondritic reference parameters. Patchett *et al.*¹³ recently argued that the chondritic ¹⁷⁶Lu/¹⁷⁷Hf and ¹⁷⁶Hf/¹⁷⁷Hf values currently in use¹⁵ could be underestimated by 3% and 2.5 ϵ -units respectively. However, because the more radiogenic ¹⁷⁶Hf/¹⁷⁷Hf signature estimated by these authors would be accompanied by a higher ¹⁷⁶Lu/¹⁷⁷Hf ratio, the chondritic parameters estimated by Patchett *et al.*¹³ would also result in initial ¹⁷⁶Hf/¹⁷⁷Hf ratios of Amitsoq zircons indistinguishable from BSE (see Supplementary Information). Hence, current uncertainties in the ¹⁷⁶Lu–¹⁷⁶Hf system are not sufficient to account for the paradoxical ¹⁷⁶Hf and ¹⁴²Nd signatures characterizing early Archaean rocks from Amitsoq.

We now turn to possible models based on magma ocean crystallization for explaining the Nd and Hf observations. A largely molten mantle would probably have been convecting too rapidly to permit extensive segregation of liquidus phases^{16,17}. As crystallization progressed, however, convection would have slowed down as the remaining crystal–magma mixture became more viscous. Under these circumstances, the timescale for residual melt extraction could approach tens of Myr (ref. 17), consistent with the estimated age of mantle depletion of 4.46 ± 0.11 Gyr (ref. 2). Thus, we anticipate that melt segregation from a crystallizing magma ocean could only have become effective at low melt fraction¹⁶. Here, we have explored the effect of extracting small amounts of melt from a crystallizing mush at the pressure of the uppermost mantle, using two models of crystallization: (1) perfect fractional crystallization, which so far has been the standard model used for evaluating the effect of magma ocean crystallization (see, for example, ref. 18); and (2) equilibrium crystallization where the melt is segregated once a given melt fraction is reached. Neither of these models is likely to be a perfect representation but they encompass the likely range of

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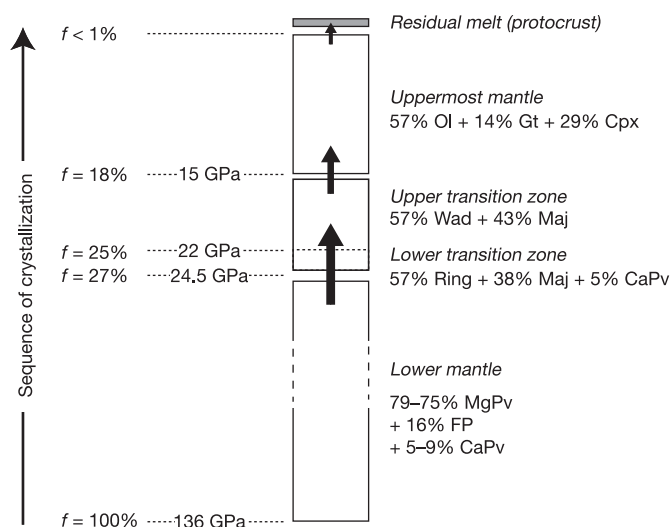


Figure 1 | Model for the crystallization of a terrestrial magma ocean. Crystallization of the magma ocean is expected to begin at lower-mantle pressures and to progress upward as temperature decreases^{16,17}. As crystallization proceeds, the residual melt is expelled to shallower depth until a small fraction of melt segregates from the mush and forms a protocrust at the surface of the Earth. Phase proportions correspond to a primitive mantle composition^{19–22}. Pressure intervals correspond to the pressures of phase transitions on the mantle solidus^{26,27}. f is the mass fraction (in wt%) of residual melt. Ol, olivine; Wad, wadsleyite; Ring, ringwoodite; Gt, garnet; Cpx, clinopyroxene; Maj, majoritic garnet; CaPv, calcium-perovskite; MgPv, magnesium-perovskite; FP, ferropericase. Crystal/melt partition coefficients for Sm, Nd, Lu and Hf are assumed to be negligible in olivine, wadsleyite, ringwoodite and ferropericase.

crystal–melt behaviours. In all cases, we began the calculation with a fully molten mantle with chondritic Lu/Hf and Sm/Nd ratios. As crystallization would proceed from the bottom up^{16,17}, we allowed the upper mantle to crystallize only when solidification of the lower mantle was complete. Similarly, at upper-mantle pressures, crystallization was considered to progress from the bottom of the transition zone upwards (Fig. 1), and calculations were divided into three discrete stages corresponding to the mineral assemblages shown in Fig. 1. Lastly, a small fraction of residual melt was allowed to segregate from the mush during the final stage of cooling, as crystallization approached 100%.

The results of the model calculations are shown in Figs 2 and 3. Figure 2 shows the Lu/Hf and Sm/Nd ratios of crystallized lower mantle and residual melt as a function of the remaining melt fraction. Figure 3 shows the Lu/Hf and Sm/Nd ratios of average upper mantle as a function of the mass fraction of residual melt removed from the mush during the final stage of solidification. The solid phase proportions were derived from refs 19–22, and partition coefficients are taken from refs 9–11, 23 and 24 (see Supplementary Information). At lower-mantle pressures, ferropericase, Mg-perovskite and Ca-perovskite are expected to crystallize over a narrow range of temperature²⁵ and therefore modal crystallization was assumed. The abundance of Ca-perovskite in the lower mantle is taken to be 5–9 wt% following estimates based on primitive mantle composition^{19–22}. As Ca-perovskite is the main mineral phase controlling fractionation of Sm/Nd and Lu/Hf at lower-mantle pressures, this enables us to place bounds on the compositions that can be generated. At pressures of the uppermost mantle, the liquidus phase would be olivine, followed by garnet and clinopyroxene²⁶. We made the assumption of modal crystallization, however, since this should be a reasonable approximation for temperatures close to the solidus, when all phases are present²⁶. In addition, as the garnet/melt partition coefficient for Lu was shown to decrease with increasing

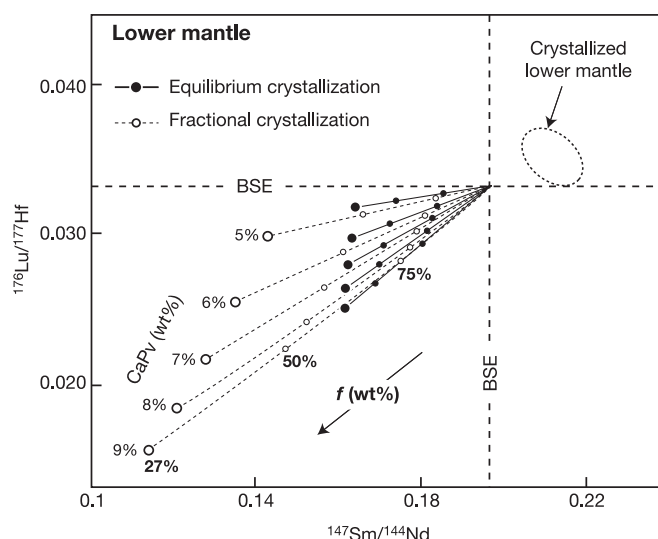


Figure 2 | Evolution of Sm/Nd and Lu/Hf ratios of residual melt during crystallization of a magma ocean at lower-mantle pressures. Lu/Hf and Sm/Nd fractionation is produced by crystallization of a lower-mantle mineral assemblage with Ca-perovskite (CaPv) abundance ranging between 5 and 9 wt%. Complete solidification of the lower mantle is attained when the mass fraction of residual melt (denoted f) reaches 27 wt% of BSE (bulk silicate Earth). The composition of the solidified lower mantle (dashed ellipse) shows little dependency on model parameters. Typical 1σ uncertainties on $^{147}\text{Sm}/^{144}\text{Nd}$ and $^{176}\text{Lu}/^{177}\text{Hf}$ ratios of the residual melt are 6.5% and 3.5%, respectively. See Supplementary Information for details of model calculations.

majorite content (and pressure)²⁴, we considered partition coefficients determined experimentally at pressures of 7 GPa (ref. 24) as representative of the uppermost mantle. In the transition zone, Lu/Hf and Sm/Nd fractionation is controlled by majorite and, above 22 GPa, by majorite and Ca-perovskite. As Ca-perovskite is expected to crystallize near the mantle solidus^{26,27}, modal crystallization may overestimate the effect of Ca-perovskite crystallization on Lu/Hf and Sm/Nd fractionation in this pressure range. We made this approximation, however, as the model curves from Fig. 3 show little dependency upon variations of Ca-perovskite abundance within a few per cent.

As shown in Fig. 2, Sm/Nd and Lu/Hf ratios in crystallized lower mantle are buffered by Ca-perovskite and deviate only slightly from chondritic values. As complete solidification of the lower mantle is expected to be faster than melt percolation¹⁶, significant amount of melt could remain trapped in the mush during solidification, which would buffer Sm/Nd and Lu/Hf fractionation even more efficiently than predicted by the model. In contrast, as melt percolation is expected to be more effective at shallower depth¹⁶, the observed fractionation of Sm/Nd ratio is more likely to reflect differentiation of the upper mantle. As shown in Fig. 3, segregation of small amounts of residual melt at upper-mantle pressures can readily generate the required mantle composition. However, the resulting Lu/Hf and Sm/Nd composition for a given melt fraction depends strongly on whether fractional or equilibrium crystallization is considered. In the fractional crystallization model (Fig. 3a), melt segregation produces more pronounced Lu/Hf fractionation in the residual mantle than does the equilibrium crystallization model (Fig. 3b), and the ‘target’ Lu/Hf fractionation is only obtained for vanishingly small amounts of melt extraction (<0.001 wt%). In contrast, equilibrium crystallization produces Sm/Nd and Lu/Hf fractionation similar to estimated values. The data are best explained by equilibrium crystallization of a lower mantle containing 7 wt% Ca-perovskite and subsequent extraction of 0.3 wt% melt from the crystallizing mush at upper-mantle pressures (Fig. 3b). This would produce

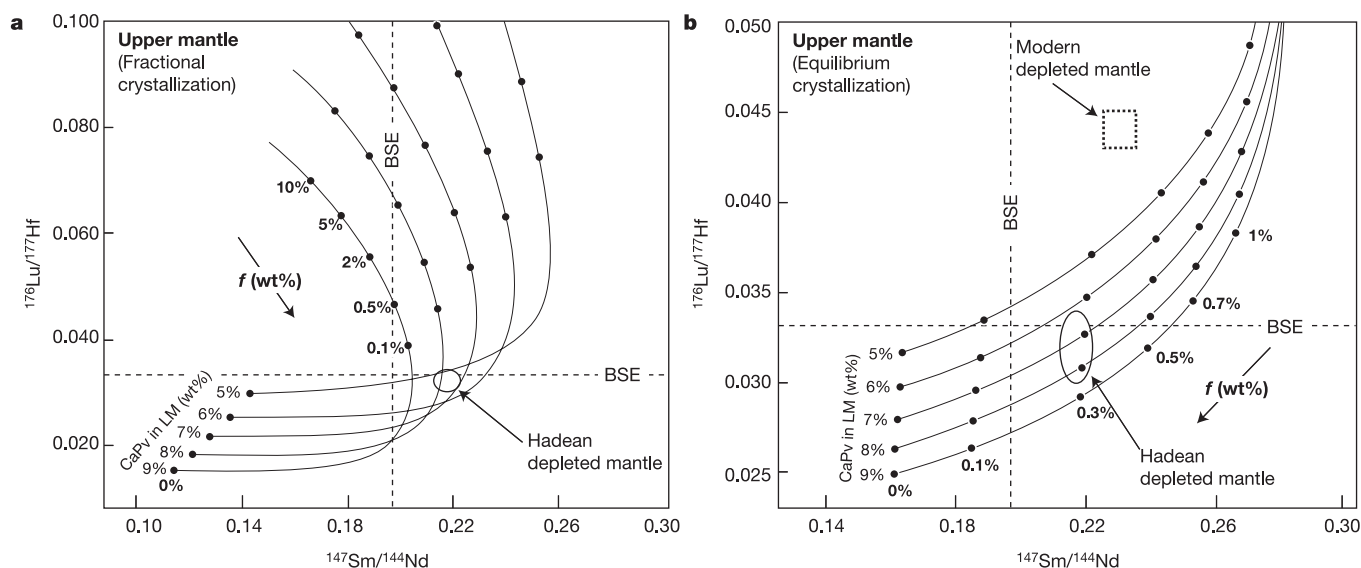


Figure 3 | Fractionation of Sm/Nd and Lu/Hf ratios generated by segregation of melt from a crystallizing magma ocean under upper-mantle conditions. **a**, Fractional crystallization, and **b**, equilibrium crystallization models for the upper mantle ($P < 24.5$ GPa). $^{176}\text{Lu}/^{177}\text{Hf}$ and $^{147}\text{Sm}/^{144}\text{Nd}$ fractionations are produced by segregation of small amounts of melt ($f = 0.1$ – 10 wt%, as labelled on the curves) from the mush during the final stage of solidification. If no melt is expelled (that is, $f = 0$ wt%), the average composition of the crystallized upper mantle is equal to the final composition of the melt shown in Fig. 2 ($f = 27$ wt%). The different curves correspond to various initial composition of the melt, following crystallization of the lower mantle (LM) with Ca-perovskite abundance

ranging between 5 and 9 wt%. The solid ellipse shows the 'target' composition for the Hadean depleted mantle. The $^{147}\text{Sm}/^{144}\text{Nd}$ ratio for Hadean mantle is estimated to be 0.217 ± 5 (1σ) from coupled $^{146,147}\text{Sm}$ – $^{142,143}\text{Nd}$ chronometry². The $^{176}\text{Lu}/^{177}\text{Hf}$ ratio of Hadean mantle is estimated to be 0.032 ± 2 (1σ) from the $^{176}\text{Hf}/^{177}\text{Hf}$ signature of 3.6–3.8 Gyr Amitsoq zircons^{5,6} (see Supplementary Information). The $^{176}\text{Lu}/^{177}\text{Hf}$ and $^{147}\text{Sm}/^{144}\text{Nd}$ ratios of modern depleted mantle are taken from ref. 29. Typical 1σ uncertainties on calculated $^{147}\text{Sm}/^{144}\text{Nd}$ and $^{176}\text{Lu}/^{177}\text{Hf}$ ratios of Hadean upper mantle are 6.5% and 4%, respectively. Model calculations are detailed in Supplementary Information.

a depleted upper mantle with superchondritic $^{147}\text{Sm}/^{144}\text{Nd}$ (0.219 ± 0.016 (s.d.)) but chondritic $^{176}\text{Lu}/^{177}\text{Hf}$ (0.0327 ± 0.0013 (s.d.)) ratios. The corresponding mass of extracted melt can be estimated to be 1.2×10^{21} kg, which would be equivalent to an ~ 8 -km-thick crust covering the Earth. Although this simplified model does not allow us to constrain the chemical composition of this protocrust, mass balance calculations indicate that it would have $^{147}\text{Sm}/^{144}\text{Nd}$ and $^{176}\text{Lu}/^{177}\text{Hf}$ ratios of 0.092 ± 0.008 (s.d.) and 0.005 ± 0.001 (s.d.), respectively, and would therefore evolve towards Hf and Nd signatures akin to upper continental crust material.

The Lu/Hf fractionation generated by fractional crystallization (Fig. 3a) is, as shown above, larger than estimated from the ^{176}Lu – ^{176}Hf system, except for unrealistically small melt fractions (< 0.001 wt%). Fractional crystallization would have been operative in the magma ocean if newly formed crystals segregated efficiently from the magma. This, in turn, would have required a significant slowdown of convective velocities, caused by high surface temperatures maintaining a low temperature gradient in the mantle over a long period of time. In theory, such an effect could be produced if the accreting Earth was covered by a thick blanketing atmosphere. Indeed, Abe¹⁷ showed that a thick atmosphere would favour chemical differentiation by inhibiting convection in the crystal–magma mixture. Given that fractional crystallization at upper-mantle pressures would produce markedly larger fractionation of Lu/Hf than observed (Fig. 3a), our results argue against a strong effect of the early Earth's atmosphere on the chemical differentiation of the magma ocean. The results of Fig. 3b indicate that the Lu/Hf and Sm/Nd signature of Hadean depleted mantle could have resulted from the solidification of a terrestrial magma ocean. This, however, requires that crystallization proceeded at equilibrium, as advocated by Solomatov and Stevenson¹⁶.

The confidence one may have in these conclusions depends partly on the accuracy of trace-element partition coefficients for Ca-perovskite. Recent studies suggest that trace-element partition

coefficients increase as the Ca content of the melt decreases during Ca-perovskite crystallization²⁸. Accordingly, crystallization of Ca-perovskite from a peridotitic melt should produce larger Sm/Nd and Lu/Hf fractionation than predicted by the model. This, in turn, would reduce the minimum amount of Ca-perovskite needed to produce the required Lu/Hf and Sm/Nd composition. Nevertheless, our results suggest that the early fractionation in the mantle indicated by ^{142}Nd anomalies and the ^{176}Hf and ^{143}Nd isotopic signatures in West Greenland rocks are best explained by late melt segregation from a crystallizing magma ocean at the end of terrestrial accretion. The absence of ^{142}Nd heterogeneity in modern oceanic basalts³ and the near-chondritic ratios of refractory lithophile elements in upper-mantle peridotites¹⁸ both strongly suggest that the terrestrial protocrust has now been efficiently remixed within the mantle, eradicating the 'fingerprints' of magma ocean crystallization. The coupled enrichments in ^{143}Nd and ^{176}Hf commonly observed in mantle-derived rocks are hence most probably inherited from extraction of continental crust later in Earth's history, at a time when the radioactive decay of ^{146}Sm had become negligible (that is, < 4.2 Gyr ago).

Received 10 March; accepted 13 May 2005.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements We thank F. G. S. Labrosse, J. Blichert-Toft and A. Halliday for discussions. Comments and suggestions by M. Rehkämper and J. Vervoort greatly improved the manuscript. This study was partly supported by the CNRS research programmes IT and PNP.

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LETTERS

Multiple volcanic episodes of flood basalts caused by thermochemical mantle plumes

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The hypothesis that a single mushroom-like mantle plume head can generate a large igneous province within a few million years has been widely accepted¹. The Siberian Traps at the Permian–Triassic boundary² and the Deccan Traps at the Cretaceous–Tertiary boundary³ were probably erupted within one million years. These large eruptions have been linked to mass extinctions. But recent geochronological data^{4–11} reveal more than one pulse of major eruptions with diverse magma flux within several flood basalts extending over tens of million years. This observation indicates that the processes leading to large igneous provinces are more complicated than the purely thermal, single-stage plume model suggests. Here we present numerical experiments to demonstrate that the entrainment of a dense eclogite-derived material at the base of the mantle by thermal plumes can develop secondary instabilities due to the interaction between thermal and compositional buoyancy forces. The characteristic timescales of the development of the secondary instabilities and the variation of the plume strength are compatible with the observations. Such a process may contribute to multiple episodes of large igneous provinces.

The time gaps between major events of the large igneous provinces (LIPs) range from a few million to several tens of million years (Fig. 1). A number of previous models have been proposed to explain the episodic plume magmatism. Plume head separation at the 660-km discontinuity¹² and a secondary instability in non-newtonian fluids¹³

could both result in the second major event of the LIPs because another plume head is formed at the original plume conduit. But these models do not generate multiple events with irregular time gaps and variable strengths in a plausible time frame. Multiple plume sources or a single dismembered plume has been considered to account for the high flux and diverse geographic distribution of magmatism generated by the Kerguelen plume during 120–95 Myr ago⁷. But multiple mantle sources that are responsible for the formation of a single LIP are not likely to be a global feature. Other models, such as solitary waves travelling along the low-viscosity, thermal plume conduit¹⁴ and the interaction of magma transport and lithospheric flexure¹⁵ are proposed to result in the discrete hotspot islands. However, it is unlikely that these mechanisms could cause such large volumes of melts as are observed in discrete LIP events.

Here we examine the evolution of the thermal plumes that entrain dense material at the base of the mantle by a series of high-resolution numerical models in an axisymmetric spherical shell. The contribution of dense material by subduction of oceanic crust (eclogite) is favoured by various observations. Seismic studies

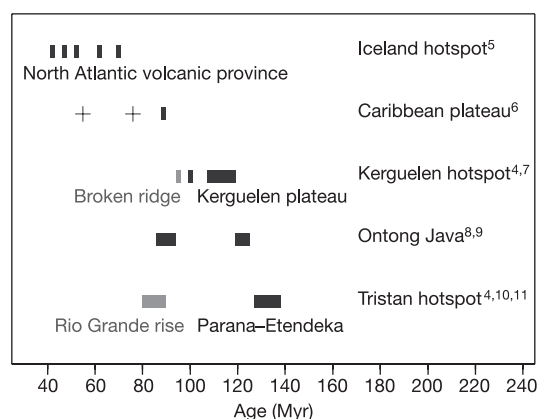


Figure 1 | Summary of the episodic major eruptions of the large igneous provinces in the last 200 million years. Time gaps between the major eruptions range from a few million to several tens of million years. The hotspot tracks for the Tristan and Kerguelen plumes are based on ref. 4. The geochronological data are mainly based on recent ⁴⁰Ar–³⁹Ar data^{5–11}. Black bars mark the major eruptions of the original large igneous provinces. Grey bars show the subsequent major eruption at a distinctive location by the same plumes due to the plate motion. Crosses represent the subsequent episodes sampled in the Caribbean plateau.

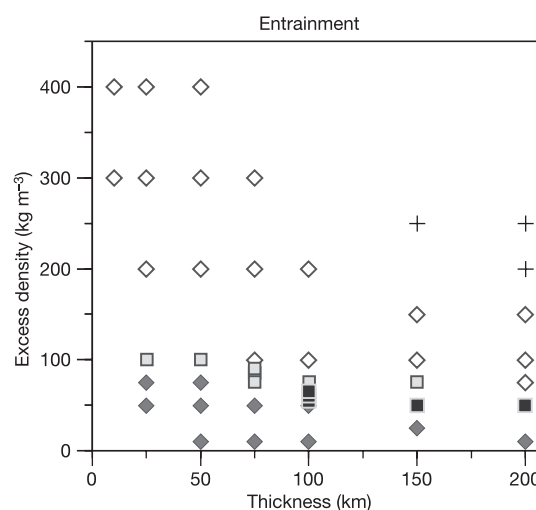


Figure 2 | Dense layer entrainment for models with $\Delta\eta = 10^2$. Crosses, stable dense layer; open diamonds, first endmember regime; filled diamonds, second endmember regime; squares, transitional regime; black squares are the models in which the secondary instabilities are developed. The density of eclogite is denser than ambient mantle everywhere except between 670–1,000 km depth^{22–24}. The dense layer is a mixture of various proportions between eclogite and ambient mantle. Other model parameters are the maximum temperature contrast across TBL (750 K), the ambient mantle viscosity (10^{22} Pa s), the reference mantle density ($4,000 \text{ kg m}^{-3}$), the thermal expansion coefficient ($3 \times 10^{-5} \text{ K}^{-1}$), and the thermal diffusivity ($10^{-6} \text{ m}^2 \text{ s}^{-1}$).

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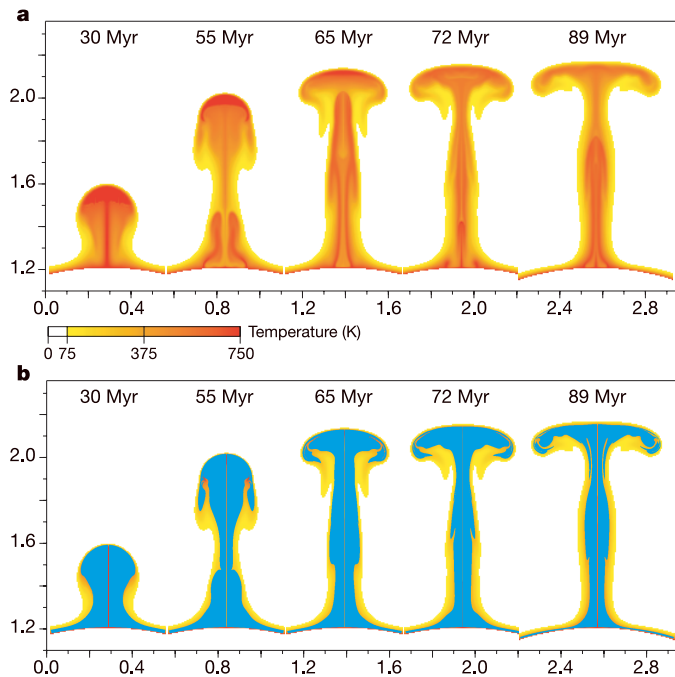


Figure 3 | Snapshots showing the evolution of the plume and the development of secondary instabilities. **a**, Thermal structure. **b**, Distribution of the dense material overlying the temperature field. The excess density is 50 kg m^{-3} with an initial thickness of 100 km for the dense layer and a viscosity contrast $\Delta\eta = 10^3$. Only the region for the plume formation is shown. Type-I instabilities are generated around $t = 55 \text{ Myr}$ and $t = 89 \text{ Myr}$. A type-II instability is generated around $t = 72 \text{ Myr}$. The length scale is normalized by the thickness of the mantle (2,885 km). A movie of this model is part of the Supplementary Information.

strongly suggest that many slabs penetrate into the lower mantle and accumulate at the base of the mantle¹⁶. Oceanic crust material will then contribute to the observed seismic heterogeneity in the lowermost mantle. The presence of the eclogite in the plume source can explain why the flood basalt is iron- and silicate-rich relative to mid-oceanic ridge basalts^{17,18}. The diverse signatures in isotopes of Nd, Pb and Sr of flood basalts can be explained by variable contributions of eclogite to the melt¹⁷. Studies of the eclogites¹⁹ and simple mass balance also suggest that a significant volume of the eclogite must enter the lower mantle. To focus on the development of the thermochemical plumes, we consider the simplified model of a single, homogeneous layer of the mixture of eclogite and lower-mantle material. This layer is initially stable at the base of the mantle but becomes perturbed by the formation of a thermal plume. Our calculations show that multiple pulses of plume material are natural consequences of a plume system with the presence of the compositionally dense material. We therefore propose that plumes

that entrain dense material can cause the discrete phases of major eruptions of flood basalt provinces.

We performed the calculations using the Sepran finite-element package modified for thermochemical convection in a Boussinesq fluid at infinite Prandtl number²⁰. The average grid resolution is $\sim 10 \text{ km}$ and the mesh is refined to 2–5-km resolution in the boundary layer and at the plume axis. The distribution of the compositionally distinctive material is modelled by the marker chain method²¹. The core–mantle boundary is represented by a free-slip, isothermal, impermeable lower boundary. The top surface and sidewall are set to be no-slip to avoid the global-scale convection so that we can focus on the evolution of the plumes. A thermal boundary layer (TBL) 130 km in thickness with a perturbation at the axis and a compositionally distinctive layer with uniform thickness is initiated at the base of the model at $t = 0$. The temperature dependence of viscosity is modelled as $\eta = \eta_0 \exp(-aT)$, where η is the viscosity, η_0 is the reference viscosity, a is a positive constant, and T is temperature. We examine models with maximum viscosity variations ($\Delta\eta$) of 10^2 and 10^3 , a reference density contrast between $0\text{--}400 \text{ kg m}^{-3}$ and dense layer thickness of 10–200 km. This parameter range covers the expected variability and uncertainties associated with the boundary layer in the lowermost mantle. Other model parameters can be found in the caption of Fig. 2 and in the Supplementary Information.

Our calculations show that the general features for the dense material entrainment by the thermal plumes can be categorized by two endmember regimes and a transitional regime (Fig. 2). In the first endmember regime, the excess density of the compositional layer is large enough to prevent plume formation from the lower portion of the TBL. The plume is formed from the upper portion of the TBL only, with consequently lower temperature and minor entrainment of dense material. This regime has been explored previously by ref. 25. In the second endmember regime the thermal effect dominates and the thermal plumes entrain significant volumes of the dense layer with high plume temperature. The regime of interest here occurs when the thermal and chemical buoyancy effects are in near-balance and secondary instabilities occur (Fig. 3).

In the intermediate regime we observe two types of instabilities (Fig. 3). The first (type I) occurs when the plume head rises; dense material drawn by the concentric flow accumulates around the base of the plume axis and induces a counter flow. Flow from the lower portion of the TBL increases the size and thermal buoyancy of the counter flow region. This region eventually becomes unstable and the type-I instability propagates at high speed through the pre-existing plume conduit ($\sim 55\text{--}65 \text{ Myr}$). The volume of this instability is of the same order of magnitude as that of the original plume head. The disappearance of the counter flow leads to the formation of a new TBL at the base of the main plume and second (type II) instability of small volume but high temperature develops ($\sim 72 \text{ Myr}$). This process can be repeated with subsequent formations of type-I ($\sim 89 \text{ Myr}$) and type-II instabilities.

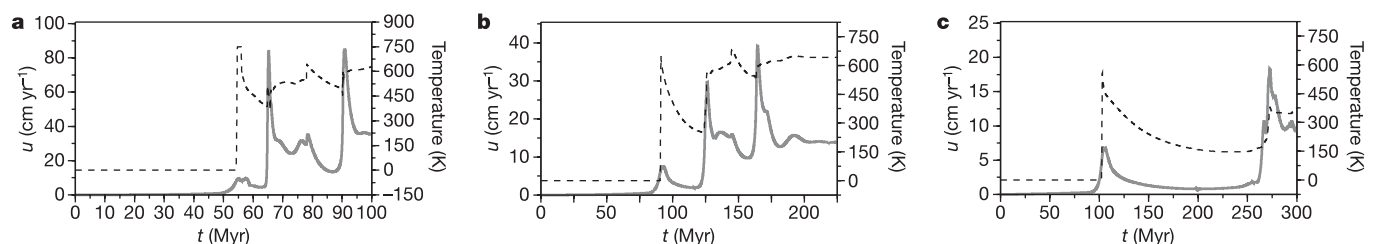


Figure 4 | Time evolution of velocity and temperature at the plume axis of 600-km depth for three representative models. Velocity, grey line; temperature, dashed line. The first peak of the velocity is generated by the original plume head. The other large peaks result from the type-I instabilities. The smaller peaks are due to the type-II instabilities. **a**, Results

for model in Fig. 3. **b**, Results for model with $\Delta\eta = 10^2$, excess density of 60 kg m^{-3} and initial thickness of 100 m for dense layer. **c**, Results for model with $\Delta\eta = 10^2$, an excess density of 50 kg m^{-3} , and an initial thickness of 200 km for dense layer.

The leading edge of the type-I instabilities propagates at an average speed of about $3\text{--}7\text{ cm yr}^{-1}$ for the model with $\Delta\eta = 10^2$ (not shown) and $5\text{--}23\text{ cm yr}^{-1}$ for models with $\Delta\eta = 10^3$ (Fig. 3). The instantaneous velocity of both types of instabilities can be as high as $10\text{--}85\text{ cm yr}^{-1}$, which is much higher than the maximum velocity of the original plume head ($5\text{--}10\text{ cm yr}^{-1}$) (Fig. 4). The high velocity of the secondary instabilities is probably a consequence of the low viscosity in the pre-existing plume conduit. It is not necessary to invoke other mechanisms such as large-amplitude solitary waves.

The temperature in the plume head initially decreases during ascent until the instabilities arrive (Figs 3, 4). The time interval between the formations of the plume head and the first instability is about 10–150 million years and that between two instabilities is 10–80 million years. The velocity of the original plume is lower than that of the instabilities. Therefore, the time separation between the arrival of the original plume head to the base of the lithosphere and the replenishment by the instabilities is generally shorter than the time interval between the formations of the plume head and the instabilities. It varies from less than 10 million to more than 100 million years. This is compatible with the time intervals of the episodic eruptions of the LIPs (Fig. 1). In addition, we find an acceptable correlation between the differences in the predicted and observed relative volumes. The estimate of the volume of the magma output inevitably involves uncertainty. Nevertheless, it is commonly accepted that the total volume of each LIP is generally greater than 10^6 km^3 . In addition, the available data shows that the magma fluxes of the later eruptions are diverse. The volume of the first major eruption is significantly larger than those of the subsequent events in the Caribbean plateau⁶ and the second major eruption is relatively small in Ontong Java^{8,9}. In contrast, the magnitudes of the later events are of the same order as that of the initial major phase of the volcanism for the Kerguelen plume⁷ and Parana–Rio Grande rise (Tristan plume)^{10,11}. Calculating melt directly from our models is complicated because it depends strongly on the structure and mineralogy of the upper mantle. Instead, we use variations in buoyancy flux as a proxy for the variations in melt volume, which we consider to be reasonable given that the melt formation in the plume head would occur under similar conditions. The time evolution of the buoyancy flux varies in our models but shows a relative range similar to that suggested by the observations (Supplementary Fig. S2).

Our models demonstrate that the multiple episodes of the major eruptions of LIPs can result from the plume with a dense layer. The excess temperature of the plume head in our model is higher than that of plumes in the upper mantle. Several mechanisms, such as the effects due to the entrainment of the surrounding mantle, the lower-viscosity upper mantle, the phase boundary at 660-km discontinuity and the layered density structure at the D'' layer, may cause this discrepancy. The viscosity of the plume is poorly known in the lower mantle, but is possibly lower than that in our models. It would lead to an even more unsteady regime and the instabilities would be induced earlier, hence reducing the time gap between the pulses of the plume material.

Received 17 January; accepted 27 April 2005.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements This research is supported in part by the National Science Foundation and the National Science Council of Taiwan, Republic of China.

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Basic avian pulmonary design and flow-through ventilation in non-avian theropod dinosaurs

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Birds are unique among living vertebrates in possessing pneumaticity of the postcranial skeleton, with invasion of bone by the pulmonary air-sac system^{1–4}. The avian respiratory system includes high-compliance air sacs that ventilate a dorsally fixed, non-expanding parabronchial lung^{2,3,5,6}. Caudally positioned abdominal and thoracic air sacs are critical components of the avian aspiration pump, facilitating flow-through ventilation of the lung and near-constant airflow during both inspiration and expiration, highlighting a design optimized for efficient gas exchange^{2,5–8}. Postcranial skeletal pneumaticity has also been reported in numerous extinct archosaurs including non-avian theropod dinosaurs and *Archaeopteryx*^{9–12}. However, the relationship between osseous pneumaticity and the evolution of the avian respiratory apparatus has long remained ambiguous. Here we report, on the basis of a comparative analysis of region-specific pneumaticity with extant birds, evidence for cervical and abdominal air-sac systems in non-avian theropods, along with thoracic skeletal prerequisites of an avian-style aspiration pump. The early acquisition of this system among theropods is demonstrated by examination of an exceptional new specimen of *Majungatholus atopus*, documenting these features in a taxon only distantly related to birds. Taken together, these specializations imply the existence of the basic avian pulmonary *Bauplan* in basal neotheropods, indicating that flow-through ventilation of the lung is not restricted to birds but is probably a general theropod characteristic.

Recent studies of non-avian theropod dinosaurs, the extinct ancestors of living birds^{11,13}, have proposed a crocodylian-like pulmonary system with a hepatic-piston model of ventilation^{14–17}. These studies counter long-held views^{9–10,12} that pneumatic vertebrae in theropods resulted from an avian-like pulmonary air-sac system (Fig. 1). Pneumatic postcranial bones are present in all clades of non-avian theropods (Fig. 2)^{9,12–13} and are typically restricted to the axial skeleton (for example, the vertebrae and ribs). Moreover, the locations of pneumatic foramina within bones are virtually identical between theropod dinosaurs and extant birds (Fig. 3). However, explicit statements about higher-level pulmonary organization and functional hypotheses for ventilating a pulmonary air-sac system in theropods have been speculative at best. Models^{18,19} proposed for the evolution of the avian pulmonary apparatus postulate a cranial to caudal developmental sequence for air sacs in the theropod lineage, with the caudal components critical for establishing flow-through ventilation predicted to occur only in derived coelurosaurs.

Correlations between regional pneumaticity and specific air sacs in both birds and theropods have long remained ambiguous^{9,10,16,17,20}, particularly with regard to the extent of the vertebral column pneumatized by the cervical air-sac system. However, results from this study indicate that regional patterns of axial pneumaticity can be unequivocally associated with specific components of the air-sac

system, and include both cervical and abdominal diverticula in addition to the lung itself. An examination of 234 air-sac-injected birds reveals the following invariant patterns: first, cervical air-sac diverticula (Fig. 1) pneumatize cervical vertebrae and ribs and the cranialmost thoracic (dorsal) vertebrae; second, abdominal air-sac diverticula invade caudal, synsacral and caudalmost thoracic vertebrae; and third, in most birds the lung itself pneumatizes adjacent thoracic vertebrae and ribs (see Supplementary Table 1). Other air sacs (Fig. 1) either do not pneumatize the skeleton (for example the caudal thoracic sac) or variably invade the sternum, sternal ribs, shoulder girdle and forelimb elements (for example the cranial thoracic, clavicular sacs). In no cases do cervical air-sac diverticula extend caudally along the column to pneumatize vertebrae beyond the middle thoracic series. This is significant because it has been asserted^{16,17} that all pneumatization of the vertebral column in birds and non-avian theropods results from cervical air-sac diverticula, thereby ignoring the dominant role of abdominal air sacs and the lung for pneumatization of the postcranial axial skeleton.

An evaluation of postcranial pneumaticity in non-avian theropods reveals consistent regional patterns similar to those of living birds. A recently recovered spectacularly preserved specimen^{12,21} of the basal neotheropod *Majungatholus atopus* (UA 8678) reveals the presence of pneumatic vertebrae in cervical, thoracic (dorsal) and sacral regions of the vertebral column (Figs 3 and 4). Pneumatic foramina in cervical vertebrae and ribs are consistent with pneumatization by cervical air-sac diverticula (Fig. 4b, f). Notably, the cervical ribs have the relatively largest pneumatic foramina known among non-avian theropods²¹. Pneumaticity of vertebral centra in the thoracic series is limited to the first four vertebrae (Fig. 4c), whereas neural arches are pneumatized throughout the entire region (Fig. 4d). This pattern of pneumaticity in thoracic centra is widespread in theropods, probably reflecting pneumatization by a firmly attached, dorsally positioned lung in the cranial half of the thorax. Pneumaticity of caudal thoracic and sacral vertebrae is restricted to neural arches and is consistent with pneumatization by abdominal air-sac diverticula (Fig. 4e). A reduction in both size and number of neural arch foramina in thoracic vertebrae 12 and 13, along with enhanced pneumaticity of sacral neural arches, indicates two different sources of pneumatization: one source for the thoracic region and one for the sacral region.

The general pattern of pneumaticity in *Majungatholus* is expressed throughout Theropoda (see Supplementary Table 1), being evident in representatives of all major neotheropod clades (Fig. 2). Sacral pneumaticity is present in at least some members of abelisauroid, spinosauroid, allosauroid, ornithomimid, tyrannosauroid and maniraptoran clades, indicating a consistent and widespread pattern of pneumatic invasion by caudally located air sacs in non-avian theropods. The postcranial pneumaticity observed in non-avian theropods implies the potential for a degree of pulmonary air sac development similar to that observed only in living birds.

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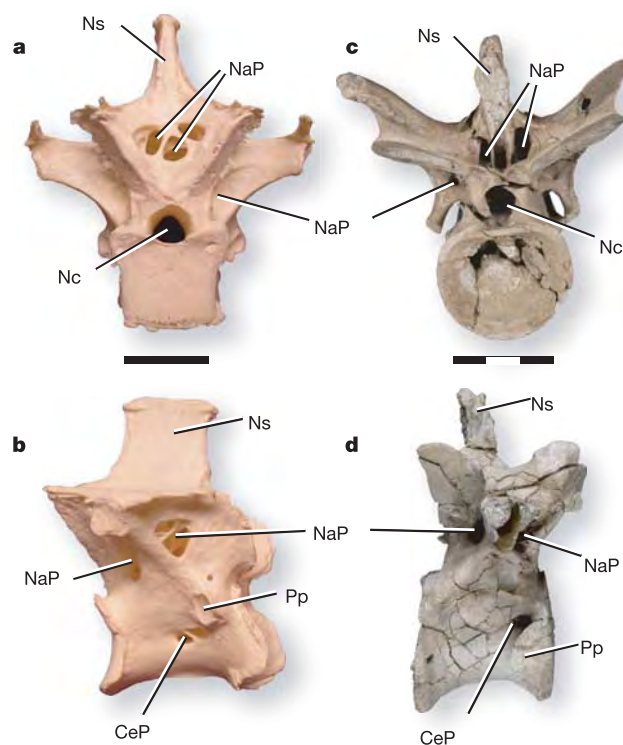


Figure 3 | Vertebral pneumaticity in avian and non-avian theropods. Comparisons between a bird (**a, b**) and theropod dinosaur (**c, d**) in caudal (**a, c**) and right lateral (**b, d**) views, illustrating the topological similarity of pneumatic features. **a, b**, Cranial thoracic vertebra of a sarus crane (*Grus antigone*, SBU AV104063). **c, d**, Mid-cervical (**c**) and cervicothoracic (**d**) vertebra of an abelisauroid theropod (*Majungatholus atopus*, UA 8678). Scale bar, 1 cm (**a, b**) and 3 cm in (**c, d**). CeP, central pneumatic foramen; NaP, neural arch pneumatic foramen; Nc, neural canal; Ns, neural spine; Pp, parapophysis.

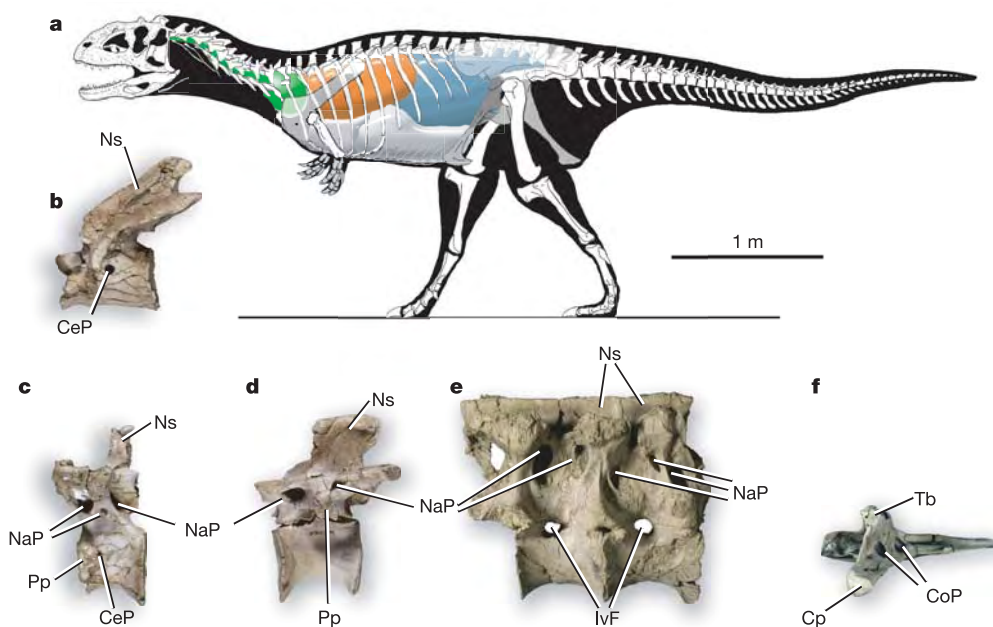


Figure 4 | Reconstruction of pulmonary air-sac system in *Majungatholus atopus* (based on UA 8678/FMNH PR 2278/2100 (ref. 21)). **a**, Pulmonary components based directly on vertebral morphology in UA 8678 include the following: cervical system (green), lung (orange) and abdominal system (blue). In modern birds, clavicular and thoracic air sacs are also present (see Fig. 1); their placement here (indicated in light grey), combined with the overlapping nature of other components, represents tertiary-level inferences emphasizing the uncertainty surrounding the reconstruction of soft tissues

apparatus. It is only when higher-compliance air sacs are positioned caudal to the lung that air will be moved through it on ventilation. An initial cranial position for air sacs, as has been predicted previously for theropods^{18,19}, would result only in expansion/contraction of the cranial air sac, and would serve little role in ventilating a caudally positioned lung. Ancillary evidence for this model comes from extant non-avian sauropsids that also possess similarly heterogeneous pulmonary systems. Although not serving ventilatory roles, nor pneumatizing the postcranial skeleton, air sacs or diverticular complexes develop on the caudal end of the lung in chameleons, varanids and snakes^{3,6}, indicating that the caudalmost region of the sauropsid lung might be relatively plastic. Sacral pneumaticity in theropod dinosaurs, the basis for inferring caudally positioned air sacs, is consistent with a 'caudal origin model' and establishes the potential for flow-through ventilation in the dinosaurian ancestors of living birds.

Flow-through ventilation of the lung provides numerous potential benefits, including increased tidal volume, decreased anatomical dead space, and airflow past gas-exchange tissues during both inspiration and expiration. High-compliance pulmonary systems also use less energy for ventilation^{2,3,5} and require relatively small adjustments of the body wall^{2,5-8}. Together these traits highlight a system with the potential for high gas exchange efficiency. Although our model does not predict the specific type of intrapulmonary air flow in non-avian theropods (unidirectional versus bidirectional), it does establish both pulmonary and skeletal prerequisites required for flow-through ventilation, a plausible early stage in the evolution of the highly derived avian pulmonary apparatus.

Recent studies of non-avian theropod dinosaurs have documented several features once thought solely to characterize living birds, including the presence of feather-like integumentary specializations²⁶, rapid, avian-like growth rates^{27,28}, and even bird-like behaviours captured in the fossil record^{29,30}. Either implicitly or explicitly, these studies have linked anatomical, physiological or behavioural inferences with an increased metabolic potential,

not constrained by osteological evidence. Unknown skeletal elements are indicated by dark grey shading. **b-f**, Vertebrae (**b-e**) and rib (**f**) of UA 8678 illustrating pneumatic features. **b**, Second cervical; **c**, first thoracic; **d**, ninth thoracic (reversed); **e**, sacral complex, left lateral view; **f**, left ninth cervical rib, medial view (reversed). CeP, central pneumatic foramen; CoP, costal pneumatic foramen; Cp, capitulum; IvF, intervertebral foramen; NaP, neural arch pneumatic foramen; Ns, neural spine; Pp, parapophysis; Tb, tuberculum.

suggesting that if not bird-like in metabolism, theropods were at least 'more similar' to birds than to reptiles. Our study indicates that basal neotheropods possessed the anatomical potential for flow-through ventilation of the pulmonary system, emphasizing the early evolution of respiratory adaptations that are consistent with elevated metabolic rates in predatory dinosaurs.

METHODS

Birds used in pulmonary studies were salvage specimens obtained from wildlife rehabilitators and museums. All cineradiographic experiments were conducted in accordance with state and institutional guidelines. A detailed taxonomic list of included taxa and pulmonary injection and cineradiographic protocols is given in the Supplementary Information.

Received 9 December 2004; accepted 4 May 2005.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements We thank M. T. Carrano, F. A. Jenkins, D. W. Krause, K. Padian, N. J. Stevens and C. S. Sullivan for comments on the manuscript; L. M. Witmer, H.-R. Duncker, A. A. Biewener, B. B. Britt, A. W. Crompton, C. A. Forster, S. F. Perry, S. D. Sampson and M. J. Wedel for discussions; the Université d'Antananarivo for permission to study UA 8678; H.-R. Duncker for access to avian preparations; R. Main and M. Daley for providing birds for cineradiographic studies; A. Milner, D. Unwin, J. Flynn, W. Simpson, P. Sereno, B. Livezey, X. Luo, M. Norell and K. Padian for providing access to specimens in their care; and R. Ridgely for assistance with Figs 1b and 4. Funding was provided by the NSF Graduate Research Fellowship Program, the Society for Integrative and Comparative Biology, an Estes Award from the Society of Vertebrate Paleontology, and the Jurassic Foundation to P.M.O.; an NSF Doctoral Dissertation Improvement Grant and Harvard University to L.C.; and the S. & D. Welles Research Fund to P.M.O. and L.C.

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Wolbachia variability and host effects on crossing type in *Culex* mosquitoes

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Wolbachia is a common maternally inherited bacterial symbiont able to induce crossing sterilities known as cytoplasmic incompatibility (CI) in insects^{1,2}. *Wolbachia*-modified sperm are unable to complete fertilization of uninfected ova, but a rescue function allows infected eggs to develop normally. By providing a reproductive advantage to infected females, *Wolbachia* can rapidly invade uninfected populations³, and this could provide a mechanism for driving transgenes through pest populations^{4,5}. CI can also occur between *Wolbachia*-infected populations and is usually associated with the presence of different *Wolbachia* strains¹. In the *Culex pipiens* mosquito group (including the filariasis vector *C. quinquefasciatus*) a very unusual degree of complexity of *Wolbachia*-induced crossing-types has been reported, with partial or complete CI that can be unidirectional or bidirectional^{6–11}, yet no *Wolbachia* strain variation was found¹¹. Here we show variation between incompatible *Culex* strains in two *Wolbachia* ankyrin repeat-encoding genes associated with a prophage region, one of which is sex-specifically expressed in some strains, and also a direct effect of the host nuclear genome on CI rescue.

Two strains of *C. quinquefasciatus* designated Bei (China) and Pel (Sri Lanka) proved to be bidirectionally incompatible with each other in a set of crossing experiments; in other words, no or very few progeny develop when Pel females are crossed with Bei males or vice versa (Fig. 1). Bei females are fully compatible with males from a subline of Pel cured of *Wolbachia* with antibiotics, designated PelU, indicating that the incompatibility between the Bei and Pel strains is induced by *Wolbachia* rather than being a result of host genetic divergence. Sequencing of the variable *wsp* gene, commonly used for

discriminating between *Wolbachia* strains¹², showed no differences between the *Wolbachia* from Bei and Pel.

The recently sequenced genome of the wMel strain of *Wolbachia* from *Drosophila melanogaster* contains an unusually high number—for a prokaryote—of genes that encode ankyrin (ANK) protein domains¹³. Genes containing these tandem motifs have been shown in other systems to mediate interactions between proteins and with the cytoskeleton, to act as transcription factors and to modify the activity of cell-cycle regulating proteins^{14,15}. Because CI seems to involve disrupting the timing of the host cell cycle at karyogamy^{16,17}, ANK genes are candidates for control of the phenotype¹³. We have sequenced the genome of the *Wolbachia* wPip strain from the *C. quinquefasciatus* Pel strain, currently at the assembly stage (http://www.sanger.ac.uk/Projects/W_pipiens/), and used BLAST searches to identify ANK genes. Primers were designed to amplify fragments from 18 different ANK genes, and were used to obtain sequences from the Bei strain.

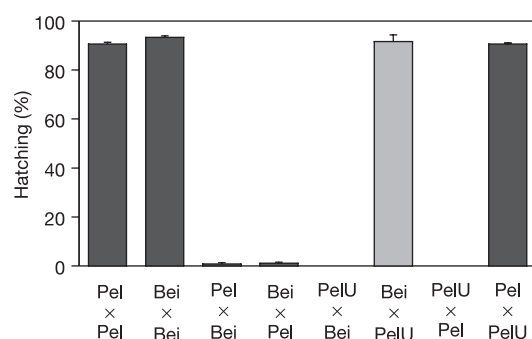


Figure 1 | *Wolbachia*-mediated CI in *C. quinquefasciatus*. Embryo hatching data is shown for crosses between the Pel, Bei and cured PelU lines. The female parents are listed first in each cross (minimum of 500 embryos counted per cross). Results are means and s.e.m.



Figure 2 | *Wolbachia* ANK gene variability. Alignment of the first 250 amino-acid residues of *pk1* (a) and all amino acids of *pk2* (b) from the incompatible Pel (upper line) and Bei (lower line) strains. Variant positions are highlighted in grey.

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Table 1 | RT-PCR amplification of *Wolbachia* genes in the prophage WO region

Strain	WO capsid	<i>pk1</i>	<i>pk2</i>
Pka females	+	+	+
Pka males	+	+	—
Pkb females	+	+	+
Pkb males	+	+	+
PelU	—	—	—

Phage capsid gene plus ANK genes were examined in adult females and adult males. Seven individuals of each sex from three Pka-group strains (Mol, Pel, Sumo) and five individuals of each sex from two Pkb-group strains (Col, Bei) were tested, plus the uninfected (cured) PelU line; all individuals within each group showed the same expression pattern.

Fifteen of the Bei DNA sequences proved to be identical to those of the Pel strain and one showed 1.2% nucleotide divergence but 0% predicted amino acid divergence from Pel, confirming the very close relationship between the *Wolbachia* in these mosquito strains. However, two genes, which will be referred to as *pk1* and *pk2*, showed significant variability in both DNA and predicted amino acid sequences (Fig. 2). *pk1*, which has nine predicted ANK domains (490 amino-acid residues in total) and is most similar to wMel WD0596 (ref. 13), showed 9.6% nucleotide divergence and 8.4% amino acid divergence between Pel and Bei in the region sequenced. *pk2*, which has three ANK domains (149 residues in total) and is most similar to wMel WD0636 (ref. 13), showed 3.1% nucleotide and 3.4% amino acid divergence between Pel and Bei. This level of variation contrasts sharply with the otherwise highly similar *Wolbachia* background.

Sequencing of *pk1* and *pk2* was also performed for six further *Culex* populations. The Sumo strain from Sri Lanka had identical *pk1* and *pk2* sequences to those in Pel; the Mol strain (*C. molestus*, from China) showed 1.1% predicted amino acid divergence in *pk1* and 0.7% amino acid divergence in *pk2* from Pel; and Col, Thai, Muh and Gzh, from Colombia, Thailand, Tanzania and China, respectively, had identical *pk1* and *pk2* sequences to those in Bei. These groupings were confirmed by the design of specific primers: Pk1a from the Pel sequence, which amplified from only the Pel, Sumo and Mol strains, and Pk1b from the Bei sequence, which amplified from only the Bei, Col, Thai, Muh and Gzh strains. Gzh and Mol had been previously shown to be unidirectionally incompatible with each other¹⁸.

Hydrophobic domains are present in *pk1* but not in *pk2*, and neither contains a predicted secretion signal. However, only 2 of 23 ankyrin-repeat proteins in wMel contained a Sec-dependent secretion signal, and it was postulated that others might be secreted through a type IV system (for which the signal is unknown)¹³. The components of this type IV system are conserved between wMel and

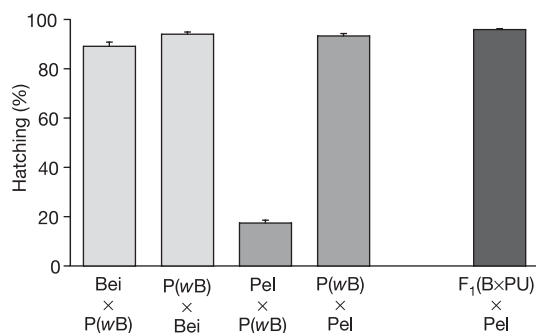


Figure 3 | Effects of host genome introgression on CI. Percentage embryo hatching in crosses between the Pel/Bei strains of *C. quinquefasciatus* and a line produced by six generations of backcrossing the Pel genome into Bei cytoplasm, designated P(wB), and the F₁ hybrids of a cross between Bei females and uninfected PelU males, designated F₁(B × PU) (female listed first, minimum of 500 embryos counted per cross). Results are means and s.e.m.

wPip, and this may be the route of export of these proteins, assuming that they function outside the *Wolbachia* cell. For *pk1*, 13 of the 20 amino-acid changes that occur within ANK domains in the region sequenced are associated with sections of the ANK domain more likely to be involved in interactions with other molecules.

Both *pk1* and *pk2* are located in prophage-like regions of the *Wolbachia* genome, WO (refs 19, 20), similar to that designated WO-B in wMel (ref. 13). Two other of the sequenced ANK genes, most similar to wMel WD0633 and WD0637, respectively, are also located within prophage-like regions but the sequenced fragments showed no predicted amino acid divergence between Pel and Bei. Phage-like particles in *Culex* have been reported in previous electron microscopy studies²¹. To examine whether phage virions are produced from the WO prophage region, a *Culex* embryo homogenate was filtered through 0.22-μm pores, which are too small to allow *Wolbachia* itself to pass, and DNA was extracted; amplification by polymerase chain reaction (PCR) was observed of a WO phage capsid protein gene^{19,20} and of *pk1* and *pk2*, but not of *wsp* or other *Wolbachia* ANK genes located outside the prophage regions. This provides clear evidence that phage particles containing the two variable ANK genes are being produced.

Reverse transcriptase PCR (RT-PCR) experiments (Table 1) revealed that the phage capsid gene was being expressed, which further supports the hypothesis that phage virions are being produced from this region. These experiments also showed that in adults *pk2* was expressed only in females in Pka strains, but in both sexes in Pkb strains. Host sex-specific expression, not previously reported for *Wolbachia* genes, provides further evidence that *pk2* might be involved in the reproductive manipulations of CI, which involves

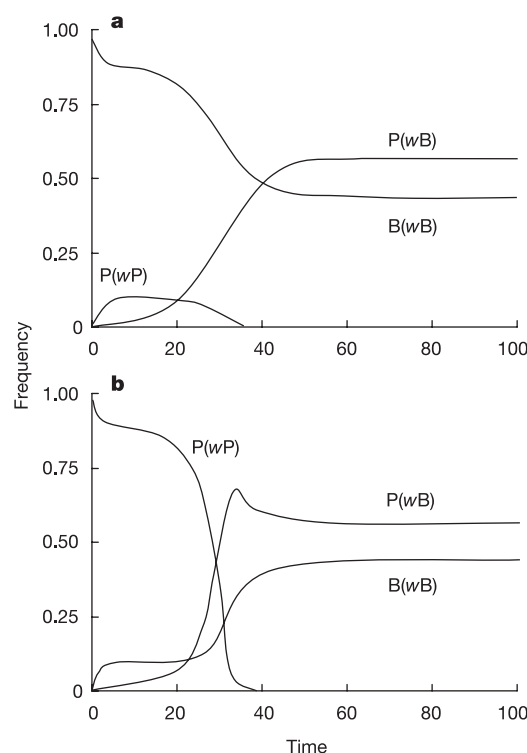


Figure 4 | Results of a model investigating host effects on CI. **a**, Population one is initially composed of B(wB) individuals; **b**, population two is initially composed of P(wP) individuals (see the text). The two populations come into contact with a migration rate of 0.09 per unit time, resulting in the generation of P(wB) individuals. The P nuclear genome allows individuals with wB *Wolbachia* to use the sperm of P(wP) males, and this results in the displacement of wP *Wolbachia*. In this model, after wP has been lost there is no difference in the fitness of P(wB) and B(wB) individuals, which remain at a neutral equilibrium.

sperm modification in males but rescue in females. That this sex specificity is seen only in some strains adds interest, given the CI observed between the Pel (Pka) and Bei (Pkb) strains.

Theoretical work has predicted that host alleles able to restore partial or full compatibility would be selected for, and thus should occur naturally^{22,23}. We therefore examined whether the host genome has any effect on crossing type by introgressing the Pel nuclear genome into a Bei cytoplasmic background. Bei females were backcrossed with *Wolbachia*-uninfected PelU males for six generations. The resulting line, designated P(wB), should have more than 99% Pel nuclear genome but should harbour Bei *Wolbachia*; this was confirmed by PCR and sequencing. There was full compatibility in both directions when it was crossed with the Bei strain (Fig. 3), but when P(wB) females were crossed with Pel males, full compatibility was also observed, in contrast to the incompatibility produced by crossing Bei females with Pel males. When Pel females were crossed with P(wB) males, only partial incompatibility was seen. This direct host effect on CI is genetically dominant, because the F₁ offspring of the Bei × PelU cross are also fully compatible with Pel males.

The host restoration of *Wolbachia*-imposed incompatibilities may be the result of a process of co-adaptation between *Wolbachia* and host, indicating that barriers to gene flow in *Culex* imposed by bidirectional incompatibilities might break down as the host genotypes introgress. Insecticide-resistance-conferring alleles have been shown to have spread worldwide in *C. quinquefasciatus* in a short time²⁴; this would be difficult to reconcile with significant barriers to gene flow associated with frequent bidirectional incompatibilities but is easier to explain in the light of the presence of host restorer alleles. Figure 4 shows a model (described in Supplementary Information) representing a natural population with *Wolbachia* type wP that is in contact through migration with a population of *Wolbachia* type wB, with complete bidirectional incompatibility between them. A host allele P allows wB females to rescue sperm from both wB and wP males. Under these conditions there is a rapid replacement of *Wolbachia* type wP with wB, through unidirectional CI, and the P allele also increases in frequency (but does not reach fixation because of the disappearance of the *Wolbachia* type wP).

Understanding the molecular basis of CI will greatly facilitate its manipulation, particularly for spreading transgenes that block the transmission of important pathogens through mosquito populations^{25–27}. The two variable prophage-associated ANK genes provide valuable markers to aid this process, together with the recently reported variability in the WO phage capsid gene²⁸, and are also highly promising candidates for a direct role in CI. An observation of crossing-type conversion after 0.23-μm pore filtration and inoculation has previously been reported in *Nasonia* wasps²⁹; phage particles containing CI genes could explain this result. Furthermore, in a *Drosophila* *Wolbachia* strain that does not express CI, the wMel gene WD0636, which is most similar to *pk2*, has been found to contain a stop codon that would result in a truncated protein (I. Iturbe-Ormaetxe and S. L. O'Neill, personal communication). *Wolbachia* incompatibilities associated with phage genes that may move horizontally more easily than *Wolbachia* itself, together with nuclear restorer alleles that can prevent the host from suffering the reproductive losses associated with CI, can together explain the long-standing unsolved problem of crossing-type complexity in *Culex*.

METHODS

Curing and crossing experiments. To create the uninfected line PelU, larvae of the Pel strain (Sri Lanka) were reared for 4 days in 50 μg ml⁻¹ rifampicin, and adults were provided with 2 mg ml⁻¹ tetracycline in cotton wool, over eight generations. *Wolbachia* infection at each generation was monitored by *wsp*-specific PCR with primers 81F/691R, plus nested PCR with primers 183F/522R (ref. 12). DNA was extracted from individual mosquitoes with the use of a Livak buffer method³⁰. Bei females were crossed with males from the line

PelU, followed by backcrossing female offspring with PelU males for a further six generations, to create the line designated PwB.

Crosses between *Culex* strains were conducted with 50 virgin individuals of each sex, and the percentage of hatched eggs was counted from individual female egg rafts 120 h after oviposition. Female spermathecae were examined for the presence of sperm if the hatching rate was low or zero, and always confirmed high rates of insemination. A total of 13,508 embryos were counted in the crosses shown in Figs 1 and 3, with a minimum of 500 per cross.

PCR and sequencing analysis. BLAST searches were used to identify ANK genes, to carry out comparative sequencing between the incompatible Pel and Bei strains. Primers were designed for 18 ANK genes, and used for PCR amplification, cloning and sequencing together with the *Wolbachia* *wsp* gene primers 81F/691R (ref. 12). Primers used to amplify the gene designated *pk1* were Pk1F (5'-AAT AAA TAC GGG TCA GAA CAA GATA-3') and Pk1R (5'-TAT CTC CTC GCG TTA GTA AAA CTTC-3') (annealing temperature 52.5°C) plus specific primers Pk1aF (5'-AGT GCC TCA TTA ATC AAC CTG GAT-3') and Pk1aR (5'-GTG CGC TAT TTA CAT CTG CTC CTA-3') (annealing temperature 52.5°C), and Pk1bF (5'-ATT TCA AAT ATA AGA GAA GAT TTT G-3') and Pk1bR (5'-AGT AAC TTT ACT ATC TCC AAC TTT TT-3') (annealing temperature 50°C), designed from the Pel and Bei sequences respectively. Primers used to amplify the gene designated *pk2* were Pk2F (5'-ATT ATG ATA AAG CTT GGT AAG AA-3') and Pk2R (5'-TTA GCC CTT CAT AAA TAG CTT-3') (annealing temperature 50°C).

To produce filtered preparations from which *Wolbachia*, but not phage particles, had been removed, about 30 embryo rafts collected within 30 min of oviposition were lightly homogenized in Livak buffer and centrifuged at 300g; the supernatant was then centrifuged at 4,000g for 5 min. The resulting pellet was resuspended in 100 μl of isotonic buffer and centrifuged at 300g for 5 min. The supernatant was then passed through a Millex GS 0.22-μm filter (Millipore) with a further 400 μl of isotonic buffer, and the filtrate was centrifuged at 20,000g at 4°C. The pellet was resuspended in 50 μl of STE buffer (0.1 M NaCl, 10 mM TrisCl, 1 mM EDTA), incubated at 95°C for 10 min and used as a PCR template with primers WO F and WO R as described^{20,21} plus primers Pk1F, Pk1R, Pk2F and Pk2R. These three primer sets were also used for RT-PCR with Qiagen One-Step RT-PCR kits, using RNA extracted from single mosquitoes with the TRI reagent (Sigma); the same primer sets were used on all the RNA extractions without reverse transcriptase to confirm the absence of contaminating DNA.

Received 23 November 2004; accepted 14 April 2005.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements We thank S. O'Neill for *Wolbachia* genomics input, and S. Song for providing *Culex* material. This research was supported by grants from the Wellcome Trust, and the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (TDR).

Author Information Sequences have been deposited with NCBI GenBank under accession numbers DQ000469–DQ000472. Reprints and permissions information is available at npg.nature.com/reprintsandpermissions. The authors declare no competing financial interests. Correspondence and requests for materials should be addressed to S.P.S. (steven.sinkins@zoo.ox.ac.uk).

Long-term sensory deprivation prevents dendritic spine loss in primary somatosensory cortex

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A substantial decrease in the number of synapses occurs in the mammalian brain from the late postnatal period until the end of life^{1–5}. Although experience plays an important role in modifying synaptic connectivity^{6–17}, its effect on this nearly lifelong synapse loss remains unknown. Here we used transcranial two-photon microscopy to visualize postsynaptic dendritic spines in layer I of the barrel cortex in transgenic mice expressing yellow fluorescent protein. We show that in young adolescent mice, long-term sensory deprivation through whisker trimming prevents net spine loss by preferentially reducing the rate of ongoing spine elimination, not by increasing the rate of spine formation. This effect of deprivation diminishes as animals mature but still persists in adulthood. Restoring sensory experience after adolescent deprivation accelerates spine elimination. Similar to sensory manipulation, the rate of spine elimination decreases after chronic blockade of NMDA (*N*-methyl-D-aspartate) receptors with the antagonist MK801, and accelerates after drug withdrawal. These studies of spine dynamics in the primary somatosensory cortex suggest that experience plays an important role in the net loss of synapses over most of an animal's lifespan, particularly during adolescence.

In the cerebral cortex of humans and other mammals, rapid synaptogenesis during early postnatal life is followed by a substantial (~50%) loss of synapses that extends through adolescence^{1–5}. In adulthood, the number of synapses continues to decline, but at a much slower rate^{1–5}. The mechanisms that underlie this extensive loss of synapses occurring throughout life are unknown. It is well established that experience profoundly influences synaptic connectivity and behaviour^{6–11}. Although experience or activity-dependent synaptic plasticity varies with experimental systems and time scales of manipulation^{6–16}, it is generally believed that experience leads to an increase rather than a decrease in the number of synapses^{8,9,17}. The influence of experience on the extensive loss of synapses that occurs from young childhood until the end of life, however, remains unknown.

To address this question, we examined the long-term effect of sensory experience on the rates of dendritic spine formation and elimination in mouse barrel cortex, a cortical region to which sensory input can be easily manipulated. Dendritic spines are the postsynaptic sites of the vast majority of excitatory axo-dendritic synapses in the brain, and their dynamism therefore serves as a good indicator of synaptic plasticity¹⁸. We used transcranial two-photon microscopy and transgenic mice that express yellow fluorescent protein (YFP) predominantly in layer V pyramidal neurons^{4,19} to repeatedly image individual dendritic branches and spines in layer I of the barrel cortex, after various periods of sensory deprivation and recovery. At the end of repeated imaging, cytochrome oxidase staining was used to confirm that the imaged area was located within barrel cortex (Fig. 1a, b).

In control mice at one month of age, we found that the percentage of spines eliminated over a two-week interval was significantly higher than the percentage of spines formed ($16.9 \pm 2.5\%$ (mean $\pm$ s.d.) elimination versus $6.3 \pm 2.3\%$ formation; 1,366 spines; $n = 5$ animals; $P < 0.01$) (Fig. 1c, d, g). Similar results have been found in other cortical regions at the same age^{4,5}, suggesting that a reduction in total spine number occurs in different cortices during this period of postnatal life. To determine the effect of sensory experience on spine turnover, we performed daily trimming of all the whiskers on one side of the facial pad from weeks 4–6, and examined the percentages of spines formed or eliminated in the barrel cortex contralateral to the trimmed side. Trimming whiskers for two weeks significantly reduced the percentage of spines eliminated compared with that in control littermates (trimmed: $9.5 \pm 1.5\%$ elimination; 1,581 spines, $n = 5$; $P < 0.01$) (Fig. 1c–g). In contrast, there was no significant difference in the percentage of spines formed between whisker-trimmed and control mice (trimmed: $5.4 \pm 1.0\%$; $P > 0.8$) (Fig. 1g). Furthermore, extending the period of whisker trimming for an additional two weeks continued to have a significant effect on spine elimination (trimmed: $7.1 \pm 2.3\%$; 550 spines; $n = 4$; control (weeks 6–8): $10.7 \pm 0.8\%$; 753 spines; $n = 5$; $P < 0.05$) but not spine formation (trimmed $4.3 \pm 1.5\%$ versus control $5.4 \pm 0.4\%$; $P > 0.4$). Thus, during the second postnatal month, when extensive spine loss occurs, sensory deprivation preferentially reduces the rate of spine elimination but not formation.

Dendrites in the developing cortex contain not only spines but also filopodia, which are long and thin protrusions without a bulbous head^{4,5,18,20} (Fig. 1c–f, i). As animals mature, filopodia become less abundant^{4,5} (Fig. 1i). Unlike spines that persist over weeks to months, filopodia undergo rapid turnover (within hours) and might serve as precursors of spines^{4,5,18,20}. In barrel cortex from one-month-old mice, we found that $9.9 \pm 3.5\%$ of dendritic protrusions were filopodia (5,388 protrusions; 18 animals) (Fig. 1i). Whisker trimming for two weeks had no significant effect on filopodia elimination ($P > 0.4$) or formation ($P > 0.8$) (Fig. 1h), even though spine elimination was significantly reduced during the same period (Fig. 1g). These results indicate that filopodia and spines have very different susceptibilities to manipulation of experience.

As mice mature into adulthood (>4 months of age), the rate of spine elimination over a two-week period is significantly reduced ($5.1 \pm 1.2\%$; 707 spines; $n = 4$) and becomes comparable to that of spine formation ($4.4 \pm 1.3\%$; $P > 0.4$). In contrast to young adolescent animals (1–2 months of age), whisker trimming for two weeks in adulthood had no significant effect on spine elimination (trimmed $4.9 \pm 1.1\%$; 547 spines; $n = 4$; $P > 0.6$) (Fig. 2i), suggesting that the effect of sensory deprivation on spine elimination decreases as animals reach adulthood. Furthermore, no significant difference in spine formation was found between control and deprived adult mice

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over two weeks ($P > 0.6$) (Fig. 2i). However, when the duration of sensory deprivation was extended from two weeks to two months, we did find a significant reduction in spine elimination (trimmed: $7.1 \pm 1.4\%$; 672 spines; $n = 5$; control: $10.2 \pm 1.6\%$; 907 spines; $n = 6$; $P < 0.01$) (Fig. 2a–h, j). No significant difference in spine formation was found between the control and trimmed groups even after two months of whisker trimming ($P > 0.7$). Thus, sensory deprivation continues to affect spine elimination in adulthood, albeit to a lesser degree than in young adolescence. The same conclusion can be generalized to all dendritic protrusions ($P < 0.05$ for elimination and $P > 0.3$ for formation), as filopodia represent only $4.2 \pm 3.3\%$ of total protrusions in adulthood (Fig. 1i; 2,916 protrusions, $n = 18$) and their number is not significantly affected by long-term deprivation.

Because more spines were eliminated than formed over weeks to months in the maturing barrel cortex, spine density in one-month-old barrel cortex (0.47 ± 0.04 spines μm^{-1} ; 933 spines; $n = 6$) is $\sim 27\%$ higher than that at six months of age (0.37 ± 0.03 spines μm^{-1} ; 845 spines; $n = 6$; $P < 0.005$). As whisker trimming preferentially reduces spine elimination in young adolescence and adulthood, we predicted that a higher spine density should be found in barrel cortex that has been subjected to long-term sensory deprivation. To test this directly, we trimmed whiskers unilaterally on a daily basis from 1–6 months of age and compared spine density of layer V pyramidal cells in layer I of barrel cortex both ipsilateral and contralateral to the whisker-trimming side. We found that spine density on the side ipsilateral to deprivation (0.36 ± 0.04 spines μm^{-1} ; 886 spines; $n = 6$) is not significantly different from age-matched, non-deprived control mice ($P > 0.4$), but that spine density is $\sim 22\%$ higher on the side contralateral to

deprivation (Fig. 2k; 0.44 ± 0.03 spines μm^{-1} ; 754 spines; $P < 0.005$). These results indicate that long-term sensory deprivation from young adolescence to adulthood leads to a significant reduction in spine loss.

To determine whether the effect of deprivation on spine loss is reversible upon sensory restoration, we first trimmed whiskers in one-month-old mice for two weeks and then allowed the whiskers to re-grow for the next two weeks. We found that during the two-week recovery period (weeks 6–8), the percentage of spine elimination exceeded that of age-matched controls, with no significant change in spine formation (Fig. 3a) (elimination: $14.9 \pm 1.7\%$ versus control $10.7 \pm 0.8\%$, $P < 0.05$; formation: $6.6 \pm 2.0\%$ versus control $5.4 \pm 0.4\%$, $P > 0.3$). Similar results were also found in mice whose whiskers were trimmed for four weeks and then allowed to re-grow in the next two weeks (Fig. 3b) (elimination: $13.5 \pm 1.6\%$ versus control $6.7 \pm 1.1\%$, $P < 0.05$; formation: $5.9 \pm 1.8\%$ versus control $5.4 \pm 1.5\%$, $P > 0.8$). In both cases, the reduction in total spine loss due to previous adolescent deprivation was largely ($\sim 80\%$) compensated for during the subsequent two-week recovery (Fig. 3c; see Methods for calculation of total spine number).

To further examine whether sensory restoration can accelerate spine elimination after animals reach adulthood, we trimmed all the whiskers on one side of the facial pad in one-month-old mice on a daily basis for five months. We found that the percentages of spine elimination and formation over the last two weeks of whisker trimming ($5.9 \pm 3.1\%$ elimination and $5.4 \pm 0.8\%$ formation; 297 spines; $n = 3$) were not significantly different from those in adult controls ($P > 0.8$ for both elimination and formation), suggesting that lack of sensory experience from young adolescence to adulthood

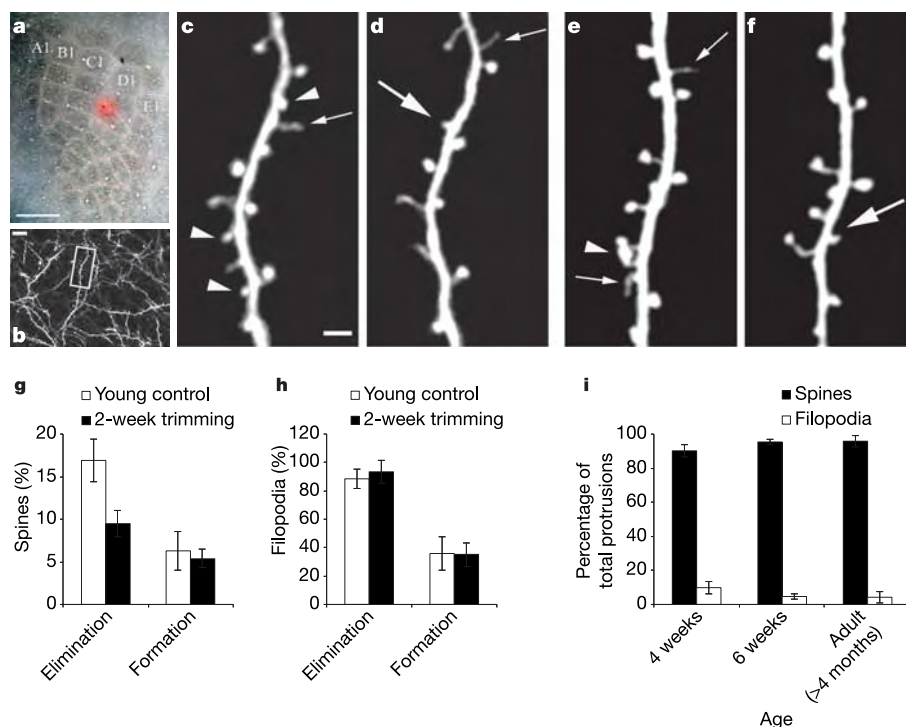


Figure 1 | Long-term whisker trimming reduces spine elimination in barrel cortex of young adolescent mice at one month of age. **a**, A cytochrome-oxidase stained tangential section indicates that the imaged region (red; labelled with DiI) is within the barrel cortex. Labels A1–E1 refer to barrels corresponding to the first rows of the mystacial pad. **b**, Low-magnification image of apical dendrites of layer V pyramidal neurons. A higher-magnification view of a dendritic segment (box in **b**) is shown in **c**. **c–f**, Repeated imaging of two dendritic branches from 4–6 weeks of age reveals spine elimination (arrowheads) and formation (large arrows) as well

as filopodium turnover (small arrows) in control (**c**, **d**) and whisker-trimmed mice (**e**, **f**). **g**, Percentage of spines eliminated or formed (number of spines eliminated or formed divided by the number of pre-existing spines) over two weeks for control and whisker-trimmed animals. Whisker trimming preferentially reduces spine elimination ($P < 0.01$). **h**, Whisker trimming for two weeks has no significant effect on filopodium turnover ($P > 0.4$). **i**, Percentages of spines and filopodia as a proportion of total protrusions at different ages. Data are presented as mean $\pm$ s.d. Scale bars, 500 μm (**a**), 10 μm (**b**), 2 μm (**c–f**).

does not prevent spines from reaching adult stability. Notably, when whiskers were allowed to re-grow and sensory experience was restored over two months, we found that spine elimination and formation are not significantly different between control and sensory-restored mice (Fig. 3d; $P > 0.8$ for both elimination and formation). Thus, although the effect of sensory deprivation on spine elimination can be largely reversed in young adolescence, it cannot be reversed

once spines are stabilized in adulthood.

Because NMDA receptor activation is essential for synaptic plasticity in a variety of systems^{21–24}, we tested the effect of chronic blockade of these receptors by applying the antagonist MK801 (0.1 or 0.25 $\mu\text{g g}^{-1}$ body weight, twice per day) for two weeks in one-month-old mice. This blockade resulted in a significant reduction in the rate of spine elimination (Fig. 4a; $P < 0.05$ for both dosages). Similar to the effects of sensory deprivation, the rate of spine formation was not significantly altered (Fig. 4a; $P > 0.5$ for both dosages). Furthermore, two weeks after the withdrawal of MK801 (0.25 $\mu\text{g g}^{-1}$ body weight), spine elimination ($15.1 \pm 2.3\%$; 682 spines; $n = 5$) was significantly increased compared with age-matched controls, and spine formation was unaffected (Fig. 4b; $P < 0.01$ for elimination and $P > 0.1$ for formation). Finally, no significant effect on either filopodia formation or elimination was observed upon NMDA receptor blockade and subsequent removal over two weeks ($P > 0.2$, for all conditions). Together, these results suggest that NMDA receptor-dependent mechanisms are involved in the extensive spine elimination during adolescence.

Our results indicate that in young adolescence, when the peak of synaptogenesis is over and synapse elimination dominates^{1–5}, long-term sensory deprivation reduces the rate of spine elimination but has no significant effect on spine formation. Furthermore, as spines become stabilized in adulthood, the effect of sensory deprivation on spine elimination is substantially reduced but still exists. As a result, sensory deprivation from 1–6 months of age increases spine density by $\sim 22\%$ after preventing spines from undergoing naturally occurring elimination (Fig. 2k). These observations underscore the importance of sensory experience in regulating the extensive loss of spines from the late postnatal period and throughout adulthood.

Owing to technical limitations, we only imaged dendritic spines of layer V pyramidal cells in layer I of the barrel cortex. Whether our results can be generalized to other neuronal types, cortical layers or

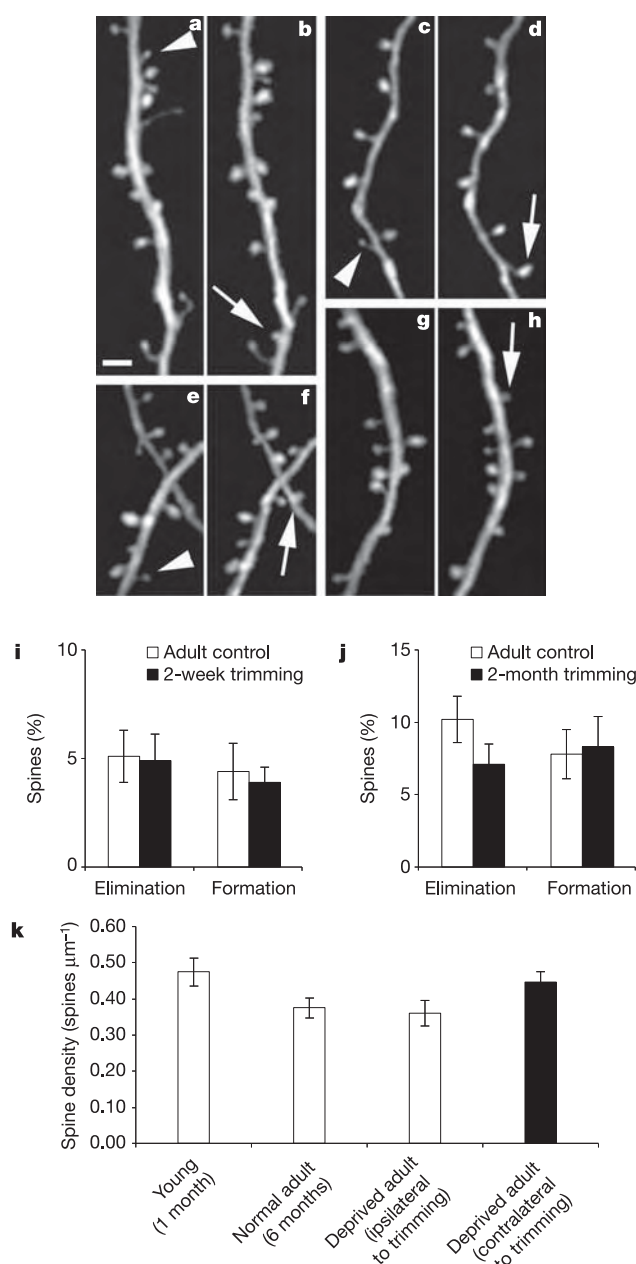


Figure 2 | The effect of long-term deprivation on spine elimination decreases but still exists in adulthood. **a–h**, Repeated imaging of dendritic branches from two normal (**a–d**) and two whisker-trimmed (**e–h**) adult mice shows elimination (arrowheads) and formation (arrows) of few spines over two months. Scale bar for **a–h**, 2 μm . **i, j**, Percentage of spines eliminated and formed over two weeks (**i**) or two months (**j**) in control and deprived adults. Whisker trimming significantly reduces spine elimination over two months ($P < 0.01$) but not over two weeks ($P > 0.6$). **k**, Spine density in one-month-old barrel cortex is $\sim 27\%$ higher than that in six-month-old adult barrel cortex. Unilateral whisker trimming from 1–6 months reduces spine loss on the side contralateral to deprivation compared with the ipsilateral side and non-deprived controls ($P < 0.005$). Data are presented as mean $\pm$ s.d.

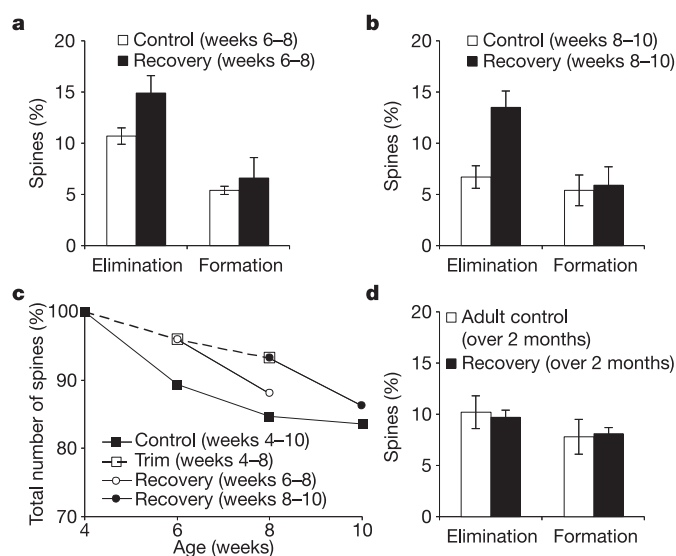


Figure 3 | Restoring sensory experience after previous deprivation accelerates spine elimination in adolescence but not in adulthood. **a**, The percentage of spine elimination, but not formation, is higher over a two-week recovery period (weeks 6–8) after a previous two-week deprivation (weeks 4–6) compared with age-matched controls ($P < 0.05$). **b**, Similar results were observed over a two-week recovery period after a previous four-week deprivation ($P < 0.05$). **c**, Percentage of total spine number from weeks 4–10 in control, trimmed and recovered animals. Whisker trimming reduces total spine loss, and sensory restoration accelerates it. **d**, Spine elimination and formation over a two-month interval are not significantly different between the control and recovered adult mice following previous deprivation from 1–6 months of age ($P > 0.8$). Data are presented as mean $\pm$ s.d.

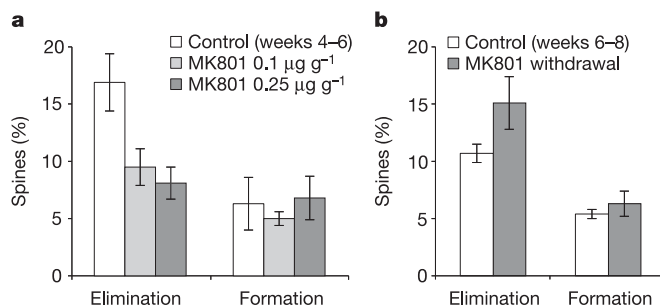


Figure 4 | Spine elimination involves NMDA receptor activation. **a**, Spine elimination, but not formation, over two weeks is significantly reduced in mice treated with MK801 (0.1 $\mu\text{g g}^{-1}$ or 0.25 $\mu\text{g g}^{-1}$ body weight, twice a day) compared with control mice ($P < 0.05$ for both doses). **b**, Spine elimination, but not formation, over two weeks is significantly increased upon MK801 withdrawal after a two-week blockade (0.25 $\mu\text{g g}^{-1}$) compared with age-matched controls ($P < 0.01$). Data are presented as mean $\pm$ s.d.

regions remains to be investigated. Activity-dependent competition and homeostatic regulation are fundamental mechanisms underlying synaptic plasticity, and both involve NMDA receptor activation^{7,8,11,13,21,23–25}. A combination of these mechanisms may be responsible for the observed effect of sensory deprivation on spine dynamics. Because of the complexity of cortical connectivity, it is possible that the effect of sensory experience on spine loss via these two mechanisms varies with cortical layers and regions. Nevertheless, as substantial synapse loss (30–50%) occurs in different cortical regions and layers in various species, including humans^{1–5}, our results raise the possibility that experience-dependent net loss of synapses is fundamental to the development and plasticity of the brain.

Previous studies have shown that synapse number in sensory cortices is reduced as a consequence of sensory deprivation during early postnatal life, and is increased in an enriched environment^{12,14,15,17,26}. These and other studies have led to the prevailing view that experience and/or neuronal activity leads to a gain rather than a loss of synapses^{8,9,17}. However, this view is not easily compatible with our findings that sensory deprivation prevents net synapse loss in the maturing mammalian brain^{1–5}. It is important to note that experience or activity-dependent modification of synapse number and strength often varies with experimental systems, synapse types and time scales of manipulation^{6,9–12,14–16,27,28} (see Supplementary Information). For example, sensory deprivation during early postnatal life results in a decrease in the number of excitatory synapses, whereas prolonged deprivation until adolescence leads to no significant changes^{14,15,26}. Our findings indicate that the effect of sensory experience on synapse number depends on the rates of synapse formation and elimination at different stages of the animal's life (Figs 1 and 2). The significant impact of adolescent deprivation on spine elimination underscores the fundamental role of childhood experience on sculpting synaptic connections. Furthermore, our observation that the effect of sensory deprivation on spine loss can only be partially reversed during adolescence (Fig. 3c) suggests that childhood experience has a long-lasting and perhaps permanent effect on later life. Experience-based pruning of synaptic connections might continue to serve as an important mechanism in learning and memory throughout adulthood^{11,29}. As sensory deprivation over periods of weeks in adulthood does not cause significant modification in spine turnover, changes in synaptic strength, but not number, might be more essential for rapid plasticity and short-term storage of information^{16,22,30}. It is worth noting that our results demonstrate long-term effects of sensory deprivation on spine dynamics in animals under laboratory housing conditions. The timescale for modifying synaptic strength and number and the degree of such modifications in complex environments and learning tasks will require future studies.

METHODS

Experimental animals. Mice expressing YFP in layer V pyramidal neurons (H-line) were purchased from the Jackson Laboratory. Mice were group-housed and bred in the Skirball animal facilities, and all experiments were done in accordance with animal protocols. Whisker trimming was performed daily by cutting the mystacial vibrissae of the right whisker-pad to skin level with a pair of scissors under a dissecting microscope. Control mice were handled under identical conditions for the same duration. The NMDA receptor antagonist MK801 (15 $\mu\text{g ml}^{-1}$ in saline; 0.1 or 0.25 $\mu\text{g g}^{-1}$ body weight) was injected twice a day for two weeks into the peritoneum of mice, starting at four weeks of age.

Surgical procedure for *in vivo* transcranial imaging and data quantification.

The procedures of transcranial two-photon imaging and data quantification have been described previously⁴. The percentage of spines eliminated or formed is defined as the number of spines eliminated or formed, divided by the pre-existing number of spines. The number of spines analysed and the percentage of spine elimination or formation under various experimental conditions are summarized in Supplementary Table S1. We calculated changes in total spine number with age as follows. The total spine number in one-month-old mice was assumed to be 100%. Percentage change in the total spine number over a given interval (for example, weeks 6–8) is relative to the animal age at the first view (for example, week 6) and calculated as the percentage of formation minus the percentage of elimination measured over that interval. Data throughout the text is presented as mean $\pm$ standard deviation (s.d.). P -values were calculated using the non-parametric Mann-Whitney U -test. Use of the Student's t -test also confirmed all the conclusions.

Determining the location of the imaged region using cytochrome oxidase staining.

At the end of repeated imaging sessions, the imaged region was identified under a dissecting microscope on the basis of CCD images of blood vasculature, and was subsequently labelled with a small lipophilic dye (DiI) crystal using a small needle tip ($\sim 100 \mu\text{m}$ in diameter). The mice were then perfused with 4% paraformaldehyde. The brains were removed, post-fixed, and tangentially sectioned into 150 μm slices using a vibratome. Sections were stained for cytochrome oxidase using a standard method.

Received 14 February; accepted 28 April 2005.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements We thank N. Kasthuri, W. Thompson and K. Helmin for critical comments on this manuscript. This work was supported by grants from the National Institutes of Health to W.-B.G.

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LETTERS

Neurosphere-derived multipotent precursors promote neuroprotection by an immunomodulatory mechanism

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In degenerative disorders of the central nervous system (CNS), transplantation of neural multipotent (stem) precursor cells (NPCs) is aimed at replacing damaged neural cells^{1,2}. Here we show that in CNS inflammation, NPCs are able to promote neuroprotection by maintaining undifferentiated features and exerting unexpected immune-like functions. In a mouse model of chronic CNS inflammation, systemically injected adult syngeneic NPCs use constitutively activated integrins and functional chemokine receptors to selectively enter the inflamed CNS. These undifferentiated cells survive repeated episodes of CNS inflammation by accumulating within perivascular areas where reactive astrocytes, inflamed endothelial cells and encephalitogenic T cells produce neurogenic and gliogenic regulators. In perivascular CNS areas, surviving adult NPCs induce apoptosis of blood-borne CNS-infiltrating encephalitogenic T cells, thus protecting against chronic neural tissue loss as well as disease-related disability. These results indicate that undifferentiated adult NPCs have relevant therapeutic potential in chronic inflammatory CNS disorders because they display immune-like functions that promote long-lasting neuroprotection.

In CNS disorders characterized by chronic inflammation (for example, multiple sclerosis, brain tumours and ischaemic stroke), transplantation of NPCs might be expected to have little therapeutic effect, as recurrent or persisting inflammation may target and destroy both CNS-resident and transplanted cells. We assessed the therapeutic potential of subventricular-zone-derived syngeneic adult NPCs (aNPCs) in a mouse model of chronic recurrent autoimmune CNS inflammation mimicking human multiple sclerosis—relapsing-

remitting experimental autoimmune encephalomyelitis (R-EAE). As a long-term consequence of repeated inflammatory episodes, neurodegenerative features such as demyelination and axonal loss are present in mouse models of R-EAE³.

SJL mice with R-EAE were injected intravenously with β -galactosidase (β -gal)-labelled aNPCs (1×10^6 cells per mouse) either at the first disease episode (13.1 ± 0.3 days post-immunization (dpi) with proteolipid protein (PLP)139–151) or at the occurrence of first clinical relapse (30.9 ± 1.1 dpi). Mice were observed for up to three months after transplantation. Clinical amelioration was observed using either treatment protocol (Table 1 and Supplementary Fig. 1). Mice transplanted with aNPCs at disease onset started to recover between 30 and 60 dpi, a period during which they underwent twofold fewer clinical relapses ($P \leq 0.05$) compared with sham-treated mice. A similarly low relapse rate was observed until the end of the follow-up period (106 dpi) ($P \leq 0.05$) compared with sham-treated mice. Mice transplanted with aNPCs at the onset of the first relapse started to recover later, but showed a threefold reduction in relapse rate between 60 and 90 dpi ($P \leq 0.005$ compared with sham-treated mice). At the end of the follow-up period, both treatment groups had a significantly lower R-EAE cumulative score and a significant reduction (58–80%) in the extent of demyelination and axonal loss compared with sham-treated mice.

Irrespective of whether aNPCs had been injected at R-EAE onset or at the first occurrence of clinical relapse, numerous β -gal-positive cells persisted within the CNS (Fig. 1 and Supplementary Fig. 2). No β -gal activity was found in the CNS of sham-treated mice (including in inflamed vessel walls) (Supplementary Fig. 2). At 106 dpi, we

Table 1 | Clinico-pathological features of R-EAE mice injected intravenously with aNPCs

Treatment	Treatment schedule	No. of mice	Disease onset (dpi)*	Maximum* clinical score	Cumulative disease score†	Relapse rate‡			Inflammatory infiltrates§ (number per mm ²)	Demyelination§ (% per mm ²)	Axonal loss§ (% per mm ²)
						0–30 dpi	30–60 dpi	60–90 dpi			
Sham	Disease onset	22	13.0 $\pm$ 0.2	2.6 $\pm$ 0.06	110.5 $\pm$ 8.1	1.4 $\pm$ 0.1	1.4 $\pm$ 0.1	1.2 $\pm$ 0.1	3.1 $\pm$ 0.5	1.4 $\pm$ 0.4	2.1 $\pm$ 0.4
aNPCs	Disease onset	13	13.1 $\pm$ 0.3	2.4 $\pm$ 0.1	64.5 $\pm$ 18.7¶	1.2 $\pm$ 0.1	0.7 $\pm$ 0.2	0.6 $\pm$ 0.2	2.04 $\pm$ 0.5	0.3 $\pm$ 0.08	0.6 $\pm$ 0.1
aNPCs	First relapse	13	14.5 $\pm$ 0.5	2.2 $\pm$ 0.08	45.6 $\pm$ 9.1#	1.8 $\pm$ 0.1	1.1 $\pm$ 0.1	0.4 $\pm$ 0.1¶	2.07 $\pm$ 0.4	0.6 $\pm$ 0.08	0.4 $\pm$ 0.07☆

*Data show mean number ($\pm$ s.e.m.).

†The cumulative score ($\pm$ s.e.m.) represents the sum of each individual score recorded for each mouse from the day of immunization (day 0) to the day on which the animal was killed (106 dpi).

‡The relapse rate represents the mean number of relapses ($\pm$ s.e.m.) per mouse occurring in the indicated period.

§Inflammatory infiltrates, demyelination and axonal loss (mean $\pm$ s.e.m.) were quantified at time of death in an average of 12 spinal cord sections per mouse for a total of five mice per group.

|| $P \leq 0.05$ compared with sham-treated controls.

¶ $P \leq 0.005$ compared with sham-treated controls.

$P \leq 0.0005$ compared with sham-treated controls.

☆ $P \leq 0.0001$ compared with sham-treated controls.

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found 2.1 ± 0.2 β -gal-positive cells per mm^2 (mean $\pm$ s.e.m.) in mice transplanted with aNPCs at disease onset, and 4.1 ± 0.5 β -gal-positive cells per mm^2 in mice transplanted with aNPCs at first relapse. The great majority of β -gal-positive cells were located around inflamed deep blood vessels of the CNS, in close contact with blood-borne, CNS-infiltrating CD45⁺ inflammatory immune cells (Fig. 1a, b), and maintained a round morphology typical of immature neural precursor cells (Fig. 1 and Supplementary Fig. 2). Some of the β -gal-positive cells showed positive staining for nestin (Fig. 1c, d; 0.28 ± 0.1 cells mm^{-2}), neuron-specific nuclear protein (NeuN) (Fig. 1e, f; 0.21 ± 0.1 cells mm^{-2}) or distal-less-related (Dlx)-2 (Fig. 1h, i; 0.12 ± 0.1 cells mm^{-2}), and a few were immunoreactive for the proneural transcription factor mammalian achaete-scute homologue (Mash)-1 (ref. 4, Fig. 1g) or for polysialylated neural cell adhesion molecule (PSA-NCAM)⁵ (Fig. 1j–m). None of the β -gal-positive cells showed immunoreactivity for NG2, glial fibrillary acidic protein (GFAP) (Supplementary Fig. 3), β -tubulin III or platelet-derived growth factor (PDGF) receptor- α . Occasionally, transplanted aNPCs showing a stream-like tubular pattern of chain migration—reminiscent of that described for migrating neuroblasts

within stem cell niches of the adult brain^{6,7}—were found in close contact with the blood vessel wall (Supplementary Fig. 2).

To better define the proliferation dynamics of aNPCs persisting within the CNS, R-EAE mice were treated with 5-bromodeoxyuridine (BrdU) at 106 dpi. A consistent fraction of transplanted β -gal-positive cells were BrdU-positive: $7.3 \pm 1.5\%$ in mice receiving aNPC transplants at disease onset, and $8.4 \pm 1.4\%$ in mice transplanted with aNPCs at first relapse (Fig. 1n). Occasionally, β -gal⁺BrdU⁺ cells expressed early differentiation markers (for example, PSA-NCAM) (Supplementary Fig. 3). A long-term study of neural stem/progenitor cell marker and BrdU labelling to prove the similarity between perivascular CNS areas in R-EAE mouse models and stem cell niches in adult brain is still lacking. However, our description of a subpopulation of intravenously injected cells that proliferate and maintain an (early) differentiated phenotype around blood vessels indicates that inflamed CNS perivascular areas in R-EAE might function as ideal, but atypical, niche-like areas in which transplanted cells can survive for a long time (up to three months after transplantation) as bona fide aNPCs.

We then turned to putative mechanism(s) that might favour aNPC

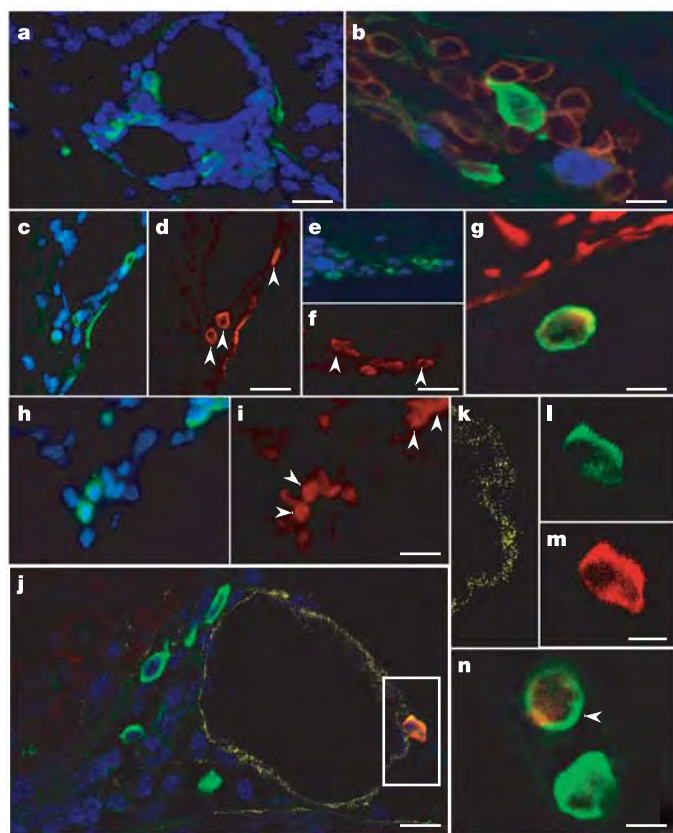


Figure 1 | aNPCs transplanted intravenously persist in inflamed perivascular areas of the CNS for up to three months after transplantation in R-EAE mice. **a, b**, β -gal-labelled, intravenously injected aNPCs (green) remain in perivascular CNS areas where blood-borne CD45⁺ immune cells (red in **b**) that form the CNS inflammatory infiltrate persist. CD45⁺ cells within perivascular areas are Ki67-negative, indicating that they are final effector encephalitogenic cells. Nuclei in **a** are in blue (DAPI). Ki67-positive cells in **b** are stained blue. **c–m**, Within perivascular areas, some of the β -gal-labelled cells (green in **c, e, g, h, j, l**) are positive for nestin (red, arrowheads in **d**), NeuN (red, arrowheads in **f**), Mash-1 (red in **g**), Dlx-2 (red, arrowheads in **i**) and PSA-NCAM (red in **j, m**). In **j** and **k**, CNS blood vessels are stained with laminin (yellow). The inset in **j** is shown in **k–m**. Nuclei in **c, e, h** and **j** are in blue (DAPI). **n**, A β -gal-labelled, intravenously injected aNPC (green) stained for BrdU (red, arrowhead). Scale bars, 10 μm (**m, n**), 15 μm (**b, g**), 20 μm (**j**), 25 μm (**f**), 30 μm (**a, d**), 40 μm (**i**).

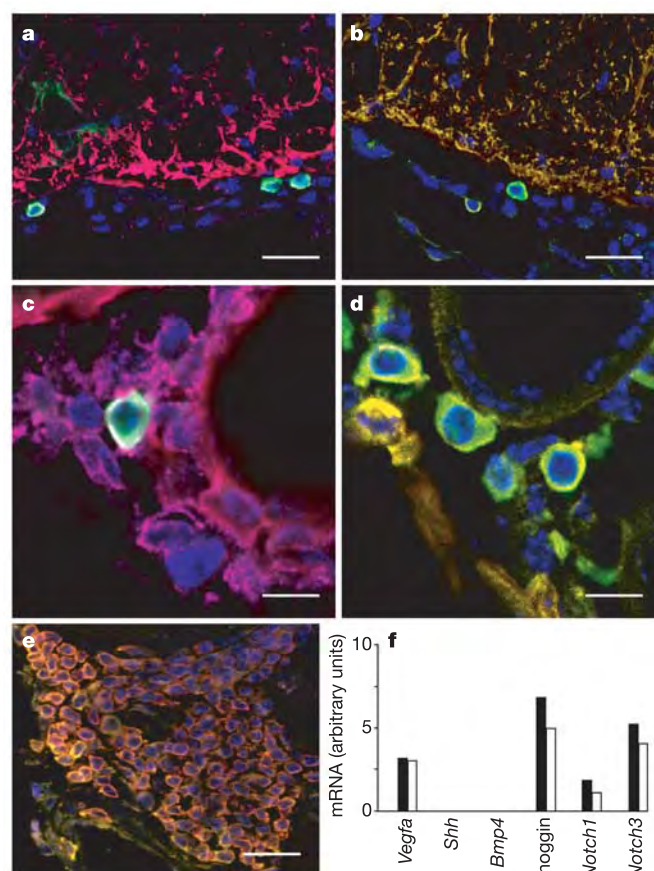


Figure 2 | Stem cell regulators co-localize with intravenously injected aNPCs that persist in perivascular areas of the CNS in R-EAE mice. **a–d**, In perivascular CNS areas, GFAP-positive astrocytes (red in **a, b**) and laminin-positive endothelial cells (red in **c, d**) that secrete BMP-4 (magenta in **a, c**) and noggin (yellow in **b, d**) co-localize with transplanted β -gal-positive aNPCs (green in **a–d**). **e**, Perivascular CNS area containing infiltrating final effector CD45⁺ lymphocytes (red) secreting noggin (yellow). Nuclei are in blue (DAPI). See Supplementary Fig. 5 for images of single staining. Scale bars, 40 μm (**a, b**), 25 μm (**c, d**), 50 μm (**e**). **f**, Real-time PCR showing mRNA levels of stem cell regulators in spleen-derived lymphocytes from naive SJL mice. Both concanavalin (Con)A-activated (black bars) and resting lymphocytes (white bars) produce *Vegfa*, *noggin*, *Notch1* and *Notch3* mRNA.

survival in inflamed perivascular CNS areas. Irrespective of aNPC transplantation, protein expression of stem cell regulators and growth factors involved both in angiogenesis and neurogenesis (for example, bone morphogenetic proteins (BMPs), noggin, notch-1, jagged-1 and vascular endothelial growth factor (VEGF)- α)^{8,9} were found in these areas until 106 dpi (Supplementary Fig. 4). BMP-4 and noggin (Fig. 2 and Supplementary Fig. 5) were secreted not only by β -gal⁺GFAP⁺ astrocytes (Fig. 2a, b) and β -gal⁺laminin⁺ endothelial cells (Fig. 2c, d), but also by blood-borne CNS-infiltrating CD45⁺ inflammatory cells (Fig. 2e). This latter result was confirmed at the messenger RNA level in spleen-derived lymphocytes (Fig. 2f).

We also investigated the cellular and molecular basis by which intravenously injected aNPCs selectively reached inflamed perivascular CNS areas in R-EAE mice. Irrespective of the mouse strain (C57BL/6 or SJL), we found that half ($53 \pm 10\%$) of aNPCs express the $\alpha 4$ subunit of the integrin very late antigen (VLA)-4 as a constitutively activated molecule organized in clusters ($12 \pm 5\%$ cells were negative for VLA-4 and $31 \pm 9\%$ had a 'dispersed'

distribution; Fig. 3a, b), a finding similar to that described in immune cells¹⁰. Spontaneous adhesion of aNPCs to purified vascular cell adhesion molecule (VCAM)-1—the VLA-4 counterligand—was basally high and comparable to that obtained with mitogen-activated CD4⁺ cells (Fig. 3c). This adhesion was not significantly increased after stimulation with chemokines such as CCL2/MCP-1, CXCL9/MIG, CXCL10/IP-10, CXCL11/I-TAC, and CXCL12/SDF-1 α (Fig. 3c). The ability of aNPCs to spontaneously adhere to VCAM-1-expressing cells was confirmed using intravital microscopy in a sub-acute C57BL/6 mouse model of brain inflammation, in which upregulation of VCAM-1 expression on microvessels was obtained by intraperitoneal injection of lipopolysaccharide (LPS)¹¹ (Fig. 3d). This finding was further supported by the specific binding of aNPCs to venules from pectoral muscle ectopically overexpressing VCAM-1 (Fig. 3e–g). A monoclonal antibody against VCAM-1 blocked more than 60% of stable aNPC adhesion (both in brain and muscle), but isotype-matched, anti-ICAM-1 and anti-MAdCAM-1 antibodies had no significant effect (data not shown).

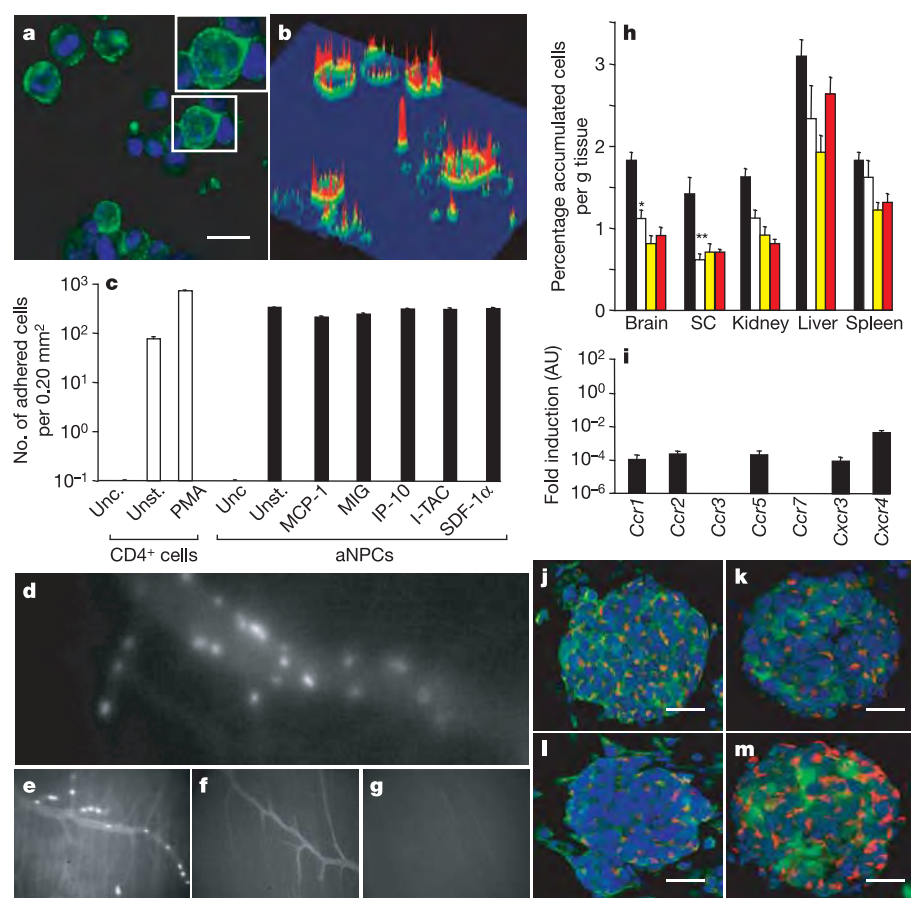


Figure 3 | aNPCs constitutively expressing VLA-4 and chemokine receptors accumulate around inflamed CNS microvessels in R-EAE mice.

a, b, Clustering (**a**, green) and 3-dimensional plot of fluorescence intensity (**b**) of VLA-4 on aNPCs. Nuclei are shown in blue (DAPI). Scale bar, 20 μ m. **c**, Unstimulated (Unst.) aNPCs and PMA-stimulated CD4⁺ T cells adhere to VCAM-1 *in vitro*. Binding does not increase when aNPCs are stimulated (for 3 min at 37 °C) with different chemokines at 1 μ M. Adhesion is completely absent in uncoated (Unc.) wells. Results expressed as mean number ($\pm$ s.e.m.) of adherent cells per 0.2 mm². **d, e**, Intravital microscopy showing aNPCs (bright intravascular dots) firmly adhering to inflamed brain (**d**) and striate muscle venules (**e**) from LPS-treated mice ($\times 20$ original magnification). **f, g**, VCAM-1 expression in inflamed striate muscle venules (**f**); isotype-matched control antibody (anti-human Ras) is shown in **g**. **h**, Accumulation of 2,3-[³H]-glycerol-labelled aNPCs in different organs

from R-EAE (black bars) and naive control (yellow bars) mice 24 h after intravenous injection. An anti-VLA-4 antibody decreases aNPC recruitment in the brain (39%) and spinal cord (SC, 54%) in R-EAE (white bars) but not in naive control mice (red bars). Asterisk, $P = 0.005$; two asterisks, $P \leq 0.0001$. Results expressed as mean percentage ($\pm$ s.e.m.) of accumulated cells per gram of tissue from four independent experiments. **i**, Mouse aNPCs express detectable levels of chemokine receptor *Ccr1*, *Ccr2*, *Ccr5*, *Cxcr3* and *Cxcr4* mRNA, but not *Ccr3* and *Ccr7* mRNA. Results are the mean of three independent experiments and show fold induction (mean $\pm$ s.d.) of mRNA levels detected in aNPCs over ConA-activated splenocytes, which were used as a positive internal control. **j–m**, Nestin-positive aNPCs (red) express CCR2 (**j**), CCR5 (**k**), CXCR3 (**l**) and CXCR4 (**m**). Receptors are stained in green in their respective panels, nuclei are in blue (DAPI). Scale bars, 80 μ m (**j–l**), 120 μ m (**m**).

To verify the relevance of this phenomenon in R-EAE mice, ^3H -glycerol-labelled aNPCs (2×10^6 cells per mouse) were injected intravenously into proteolipid protein (PLP)139–151-immunized SJL mice at the time of the first disease episode. Within 24 h of cell injection, aNPCs were found in various bodily organs, and $3.1 \pm 0.2\%$ of the cells had accumulated in the CNS (Fig. 3h). A significant reduction in aNPC recruitment to the CNS ($39\text{--}54\%$ reduction; $P \leq 0.005$) was observed after *in vitro* pre-treatment of aNPCs with an anti-VLA-4 blocking antibody (Fig. 3h). This effect was EAE-specific, as VLA-4 blocking did not result in decreased cell recruitment into the CNS (and other peripheral organs) of naive mice injected intravenously with labelled aNPCs (Fig. 3h). These results were also confirmed in a chronic progressive model of EAE in C57BL/6 mice (Supplementary Fig. 6).

Our *in vitro* studies showed that pro-inflammatory chemokines do not further activate basal avidity for counterligands of VLA-4 expressed on aNPCs (Fig. 3c). However, we cannot exclude the possibility that culturing cells with mitogens might somehow influence VLA-4 basal avidity on aNPCs *in vitro*. G-protein-coupled receptors (GPCRs) might be necessary for further increasing aNPC transendothelial migration or for mediating environmental positioning under suboptimal conditions, such as those that might occur *in vivo*^{12,13}. We found that aNPCs express a wide range of pro-inflammatory chemokine receptors, at both mRNA and protein levels. Most cells within neurospheres express the chemokine receptors CCR1, CCR2, CCR5, CXCR3 and CXCR4, but do not express CCR3 or CCR7 (Fig. 3i–m). CCR1, CCR5 and CXCR4 were functionally active, as aNPCs migrated in a dose-dependent manner in

response to the chemokines CCL5/RANTES and CXCL12/SDF-1 α (Supplementary Fig. 7). Pre-treatment of aNPCs with the G $_i$ -protein inhibitor pertussis toxin completely inhibited this migration response, showing that it was GPCR-dependent (Supplementary Fig. 7). Thus, aNPCs might use GPCRs and cell adhesion molecules to increase their migration towards inflamed lesions in the CNS. This explains the partial inhibition of aNPCs extravasation obtained in R-EAE mice using an anti- α 4-integrin blocking antibody.

We propose that inflammation acts as the 'danger signal' that determines both selective recruitment and long-term survival of intravenously injected aNPCs in the CNS of R-EAE mice. However, the mechanism(s) by which transplanted aNPCs act to protect mice from R-EAE remains to be identified. Apoptosis is considered one of the principal mechanisms for promoting recovery from EAE, by inducing programmed cell death of CNS-infiltrating encephalitogenic T cells^{14,15}. As early as two weeks after aNPC transplantation (30 dpi), R-EAE mice receiving transplants at disease onset showed a significant reduction in the number of inflammatory infiltrates in the CNS ($P \leq 0.01$) and a threefold increase in the number of CNS-infiltrating CD3 $^{+}$ TUNEL $^{+}$ cells ($P \leq 0.005$ compared with sham-treated mice) (Fig. 4a and Supplementary Fig. 8). In both aNPC- and sham-treated mice, the majority ($83.8 \pm 6.9\%$ and $90.5 \pm 2.3\%$, respectively) of apoptotic cells were confined to CNS perivascular inflammatory infiltrates. These results were confirmed using active caspase-3 as a marker for apoptosis, and also showed that transplanted β -gal-positive cells in inflamed perivascular CNS areas did not undergo apoptosis (Fig. 4b). At 50 dpi, fluorescence-activated cell sorting (FACS) analysis of CNS-infiltrating CD3 $^{+}$

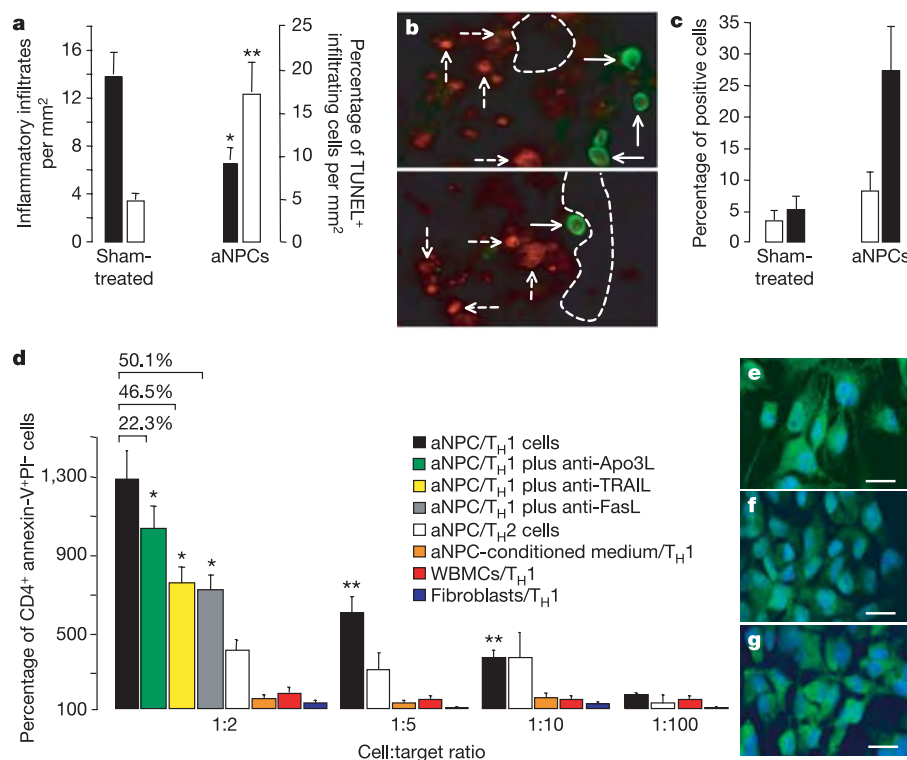


Figure 4 | aNPCs induce apoptosis of encephalitogenic T cells *in vitro* and *in vivo*. **a**, Decrease in spinal cord inflammation (black bars) and increase in CNS-infiltrating apoptotic lymphocytes (white bars) in R-EAE mice 15 days after aNPC injection (30 dpi) (mean $\pm$ s.e.m.). Asterisk, $P \leq 0.01$; two asterisks, $P \leq 0.005$ compared with sham-treated mice. **b**, Spinal cord perivascular area stained for caspase-3 (red, dashed arrows) and β -gal (green, solid arrows). Dashed lines indicate blood vessels ($\times 100$ original magnification). **c**, FACS analysis showing a significant increase in late apoptotic (TOPRO3 $^{+}$ AnnexinV $^{+}$, black bars) but not necrotic (TOPRO3 $^{+}$ AnnexinV $^{-}$, white bars) CNS-infiltrating CD3 $^{+}$ T cells in R-EAE

mice 35 days after aNPC injection (50 dpi). Results expressed as mean percentage of positive cells ($\pm$ s.e.m.) from three independent experiments. **d**, aNPCs induce apoptosis (AnnexinV $^{+}$ PI $^{-}$ cells) of PLP139–151-specific T $_H$ 1 but not T $_H$ 2 cell lines (two asterisks, $P \leq 0.05$ versus basal levels). Inhibition of FasL, Apo3L or TRAIL significantly reduces the aNPC-mediated pro-apoptotic effect (asterisk, $P \leq 0.01$). Results (mean $\pm$ s.e.m.) from three independent experiments. **e–g**, Fluorescence images showing expression of death receptors FasL/CD95-ligand (e), Apo3L (f) and TRAIL (g) on aNPCs conditioned with pro-inflammatory cytokines. Nuclei are in blue (DAPI). Scale bars, 15 μ m.

cells showed a significantly ($P = 0.01$) higher percentage ($27.3 \pm 10.4\%$) of annexin-V⁺TOPRO-3⁺ 'late apoptotic' cells in R-EAE mice transplanted at onset compared with sham-treated mice ($5.2 \pm 0.6\%$) (Fig. 4c).

In the same mice, CNS-infiltrating CD3⁺ cells did not show any sign of immunological anergy, as indicated by non-significant differences in the percentages of cells producing interferon (IFN)- γ ($17.6 \pm 2.26\%$ sham-treated versus $12.2 \pm 3.53\%$ aNPC-treated) or interleukin (IL)-2 ($17.8 \pm 1.64\%$ versus $33.6 \pm 10.7\%$), or those producing both IFN- γ and IL-2 ($59.3 \pm 1.96\%$ versus $41.0 \pm 15.7\%$). The pro-apoptotic effect of aNPCs on T cells was also confirmed by *in vitro* analyses. Spleen-derived CD3⁺ cells from naive syngenic SJL mice that had been activated by plastic-immobilized anti-CD3/CD28 antibody underwent apoptosis when co-cultured with increasing numbers of single-cell dissociated aNPCs. In a transwell system designed to prevent cell-to-cell contact, the pro-apoptotic effect of aNPCs on CD3⁺ cells was less intense but still measurable (Supplementary Fig. 8). Furthermore, *in vitro* generated antigen (PLP139–151 peptide)-specific CD4⁺ T-cell lines with a pro-inflammatory type 1 helper (T_H1) cytokine profile (for example, secreting tumour necrosis factor (TNF)- α and interferon (IFN)- γ), but not PLP139–151-specific CD4⁺ T-cell lines with an anti-inflammatory T_H2 cytokine profile (for example, secreting IL-4), underwent apoptosis when co-cultured *in vitro* with increasing numbers of single-cell dissociated aNPCs (Fig. 4d). However, fibroblasts, whole bone marrow cells or aNPC-conditioned medium did not induce measurable pro-apoptotic effects when co-cultured with PLP139–151-specific T_H1 cells.

In vitro and *in vivo* data suggest that aNPC-mediated apoptosis might be induced through both (extrinsic) death receptor- and (intrinsic) mitochondrial-mediated pathways (see ref. 16 for a review). Blockade of death receptor ligands, but not of IFN- γ and inducible nitric oxide synthase (iNOS), significantly decreased apoptosis of PLP139–151-specific T_H1 cells (22.3 – 50.1% ; Fig. 4d), indicating involvement predominantly of the death receptor pathway. However, we still cannot exclude involvement of the mitochondrial apoptotic pathway. In fact, aNPCs conditioned *in vitro* with pro-inflammatory cytokines (such as TNF- α , IFN- γ and IL-1 β)¹⁷ greatly increased (at both protein and mRNA levels) not only the membrane expression of death receptor ligands (for example, FasL, Trail and Apo3L) (Fig. 4e–g) but also the production of soluble factors potentially involved in mitochondrial-mediated apoptosis, including iNOS, IFN- γ , glial-derived neurotrophic factor (GDNF) and leukaemia inhibitory factor (LIF)^{15,16,18,19} (Supplementary Figs 8 and 9). Thus, our results indicate that aNPC-mediated T-cell apoptosis can occur via multiple distinct pathways, with the death receptor-mediated pathway probably the predominant one.

Finally, at 106 dpi we found increased numbers of process-bearing CD11b⁺ activated microglial cells within perivascular CNS areas from aNPC-treated R-EAE mice (79.7 ± 10.8 cells mm⁻² in mice treated at onset, 58.6 ± 5.6 cells mm⁻² in mice treated at first relapse, 3.3 ± 0.8 cells mm⁻² in sham-treated mice, $P < 0.001$; Supplementary Fig. 4). These microglial cells were found capable of producing pro-apoptotic substances upon *in vitro* activation with pro-inflammatory cytokines (Supplementary Fig. 8). We must therefore consider the possibility that CNS-resident glial cells might also contribute to promote T-cell apoptosis, as previously suggested²⁰.

Here we demonstrate that undifferentiated aNPCs can promote brain repair upon systemic injection into a mouse model of multiple sclerosis through previously unidentified immune-like functions. This observation is consistent with earlier reports indicating that transplanted aNPCs promote brain repair as they acquire a terminally differentiated phenotype *in vivo*^{2,21}. Our data indicate that the CNS microenvironment dictates the fate (and consequently, the therapeutic efficacy) of systemically transplanted aNPCs. When neurodegeneration prevails, transplanted cells acquire a mature functional phenotype and thus replace damaged neural cells^{2,21,22}.

As we show here, when neuroinflammation predominates, transplanted aNPCs survive recurrent inflammatory episodes by retaining both an undifferentiated phenotype and the ability to proliferate. In this situation, inflammation represents the key danger signal for orchestrating recruitment to and long-term persistence of aNPCs in areas of CNS damage. Recruitment is possible because of the innate capacity of circulating aNPCs to use selected molecular pathways (for example, integrin- and chemokine-mediated homing) used by blood-borne-derived lymphocytes in patrolling the CNS. Long-lasting persistence of aNPCs in the CNS is due to continuous cross-talk between transplanted aNPCs and inflammatory CNS-infiltrating T cells within perivascular niche-like areas, together with CNS-resident cells that produce gliogenic and neurogenic regulators. In these areas, aNPCs survive as undifferentiated cells and exert neuroprotective effects by inducing programmed cell death of blood-borne, CNS-infiltrating pro-inflammatory T_H1 (but not anti-inflammatory T_H2) cells^{23,24}.

METHODS

Adult NPC derivation and cultures. Cultures of adult NPCs were established from the subventricular zone of brains from 6–8-week-old SJL and C57BL/6 mice, as previously described². Details are provided in the Supplementary Methods.

R-EAE induction. SJL mice (Charles-River) were immunized with 200 μ g PLP139–151 (Espikem) in complete Freund's adjuvant (CFA) as described³. Body weight and clinical score (0 = healthy; 1 = limp tail; 2 = ataxia and/or paresis of hind limbs; 3 = paralysis of hind limbs and/or paresis of forelimbs; 4 = tetra paralysis; 5 = moribund or dead) were recorded daily. Clinical relapses were defined as the occurrence of an increase in clinical score of 0.5 persisting for a minimum of three consecutive days.

aNPC cell transplantation. β -gal-positive aNPCs were injected intravenously through the tail vein². Sham-treated age-, sex- and strain-matched mice injected intravenously with PBS alone were used as controls. To assess the *in situ* proliferation of intravenously injected aNPCs, mice were treated intraperitoneally with bromodeoxyuridine (BrdU, Roche, 50 mg kg⁻¹) at 106 dpi for three consecutive days and killed soon thereafter, as previously described²⁵. Details are provided in the Supplementary Methods.

Neuropathology. The brain and spinal cord of mice were removed and processed for pathology. Either paraffin-embedded or frozen tissue samples were used. Detailed descriptions of procedures and the list of antibodies are provided in the Supplementary Methods.

Immunocytochemistry. Single-cell dissociated or sphere-aggregated aNPCs were plated at 3×10^4 cells cm⁻² onto matrigel-coated glass chamber slides in growth medium and incubated for 1 h at 37°C. Fluorescent samples were analysed using a BioRad MRC 1024 confocal image microscope. Image Pro plus software was used for VLA-4 distribution analysis. The complete list of antibodies is provided in the Supplementary Methods.

***In vivo* distribution of aNPCs.** aNPCs (2×10^6 cells per mouse) were incubated for 4 h at 37°C in growth medium containing 100 μ Ci ml⁻¹ 2,3-[3H]glycerol (MP Biomedicals) and then injected intravenously into (1) C57BL/6 mice that had been immunized with 200 μ g myelin oligodendrocyte glycoprotein (MOG)35–55 (Espikem) in CFA containing 500 ng pertussis toxin² or (2) SJL mice that had been immunized with PLP139–151 (ref. 3). Mice were injected soon after disease onset and killed 24 h after transplantation. After perfusion, brain, spinal cord, kidney, spleen and liver were collected, weighed and sonicated, and the radioactive content of the tissues measured in a β -counter (LS1801; Beckman Coulter) as previously described²⁶. Data are expressed as mean percentage ($\pm$ s.e.m.) of accumulated cells per gram of tissue from a minimum of six mice per group from four independent experiments.

Static adhesion assay. aNPCs were added to 18-well glass slides at 7×10^4 cells per well in 25 μ l, and coated overnight at 4°C with (1μ g ml⁻¹) purified mouse VCAM-1 (RAND D, gift from E. Butcher) as previously described¹⁰. Spleen-derived CD4⁺ T cells (7×10^4 cells per well in 25 μ l) stimulated for 10 min with the mitogen phorbol 12-myristate 13-acetate (PMA, 100 ng ml⁻¹) were used as positive controls. The pro-inflammatory chemokines CCL2/MCP-1, CXCL9/MIG, CXCL10/IP-10, CXCL11/I-TAC or CXCL12/SDF-1 α were immediately added (1μ M) to the cell-containing wells for 3 min at 37°C. Site density per square micrometre of immobilized VCAM-1 was calculated and data are expressed as mean numbers ($\pm$ s.e.m.) of adherent cells from four independent experiments.

Chemotaxis assay. The migration response of aNPCs to different chemokines was evaluated using a modified 48-well microchemotaxis Boyden chamber

system (Neuro Probe) as previously described²⁷. Details are provided in the Supplementary Methods.

Apoptosis experiments. Spleen-derived CD3⁺ cells from naive SJL mice or encephalitogenic CD4⁺ PLP139–151-specific T-cell lines were co-cultured *in vitro* with titrated concentrations of aNPCs for 18 h at 37 °C, 7% CO₂. T cells were then harvested and FACS analysis was performed using appropriate antibodies to quantify apoptotic and necrotic CD3⁺ cells (see Supplementary Methods for a complete list of antibodies and reagents). *In vitro* pre-treatment (30 min at 18–25 °C) of aNPCs with antibodies blocking death receptor ligands was performed using hrTRAIL-R2:Fc (10 µg ml⁻¹), mrFn14:Fc (10 µg ml⁻¹) or Fas:Fc antibodies (20 µg ml⁻¹) (all from Alexis). IFN-γ and iNOS were also blocked before performing apoptosis experiments, using either a rabbit anti-mouse IFN-γ blocking antibody (Pharmingen, 10 µg ml⁻¹ for 30 min at room temperature) or by adding the iNOS inhibitor 5-methylisothiourea sulphate (Santa Cruz, 500 µM) to the co-culture wells. Data are expressed as mean percentage of positive cells over basal cells (±s.e.m.) of at least three mice per group from a minimum of three independent experiments for each protocol.

Additional experiments. The Supplementary Methods contain details regarding FACS, statistical analysis and protocols for polymerase chain reaction with reverse transcription (RT-PCR), together with lists of antibodies, cells and cell lines used.

Received 10 January; accepted 11 May 2005.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements We wish to thank R. Galli, A. Gritti, M. Muzio, A. Vescovi and L. Ricci-Vitiani for discussions. We are grateful to F. Mavilio for critically discussing the manuscript. We acknowledge the technical help of S. Bach, S. Bucello, E. Butti, C. Covino, R. Molteni, A. Palini and C. Sciorati. S.P. is the recipient of a fellowship from the National Multiple Sclerosis Society (NMSS). L.Z. is the recipient of a fellowship from The Myelin Project (TMP). This work was supported in part by the Italian Minister of Health, the Italian Multiple Sclerosis Foundation (FISM), NMSS and TMP.

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LETTERS

Cancer gene discovery in solid tumours using transposon-based somatic mutagenesis in the mouse

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Retroviruses, acting as somatic cell insertional mutagens, have been widely used to identify cancer genes in the haematopoietic system and mammary gland^{1,2}. An insertional mutagen for use in other mouse somatic cells would facilitate the identification of genes involved in tumour formation in a wider variety of tissues. Here we report the ability of the *Sleeping Beauty* transposon to act as a somatic insertional mutagen to identify genes involved in solid tumour formation. A *Sleeping Beauty* transposon, engineered to elicit loss-of-function or gain-of-function mutations, transposed in all somatic tissues tested and accelerated tumour formation in mice predisposed to cancer. Cloning transposon insertion sites from these tumours revealed the presence of common integration sites, at known and candidate cancer genes, similar to those observed in retroviral mutagenesis screens. *Sleeping Beauty* is a new tool for unbiased, forward genetic screens for cancer genes *in vivo*.

DNA 'cut-and-paste' transposons have recently been developed for use in vertebrates. Dormant *Tc1/mariner*-family transposable elements in the genomes of salmonid fish have been identified³. Directed mutagenesis was used to correct mutations that had silenced the activity of the transposases. A resurrected transposable element, called *Sleeping Beauty* (SB), was shown to be active *in vivo*³. SB functions as a germline insertional mutagen^{4–6}, but the use of SB in the mouse soma has previously been limited to gene therapy studies^{7,8}. Here we show that chromosomally resident SB transposons can contribute to cancer development when mobilized in the soma of SB10 transposase transgenic mice.

A SB transposon, called T2/Onc, was engineered to induce both loss-of-function and gain-of-function mutations (Fig. 1a). T2/Onc contains splice acceptors followed by a polyadenylation signal in both orientations to intercept upstream splice donors after intronic insertion to generate loss-of-function mutations. Between the two splice acceptors are sequences from the 5' long terminal repeat (LTR) of the murine stem cell virus (MSCV) that contain strong promoter and enhancer elements that have been shown to be active in stem cells^{9–11}. Immediately downstream of the LTR is a splice donor for splicing of a transcript initiated from the LTR into downstream exons of endogenous genes. Two lines (nos 68 and 76) of T2/Onc transgenic mice were used for analysis (see Methods).

The ability of T2/Onc to mobilize in the soma was tested by breeding T2/Onc transgenic animals to transgenic mice expressing the SB transposase regulated by the ubiquitous CAGGS promoter¹² (CAGGS-SB10)⁴. Excision of T2/Onc from the concatemer was detected in every somatic tissue tested from T2/Onc;CAGGS-SB10

doubly transgenic animals but not in tissue from singly transgenic controls (Fig. 1b), whereas Southern blotting of normal tissue revealed few or no clonal, somatically acquired, T2/Onc insertions (Supplementary Fig. 1a). New subclonal T2/Onc insertions ($n = 12$) could be cloned from doubly transgenic somatic genomic DNA (data not shown). These data showed that SB transposition occurs readily in somatic cells.

Mice doubly transgenic for both T2/Onc and CAGGS-SB10 were aged for more than 1 year ($n = 26$) but did not show evidence of cancer susceptibility different from background (data not shown). We proposed that somatic T2/Onc mobilization by CAGGS-SB10 alone is insufficient to promote rapid, highly penetrant tumour formation in wild-type animals but might accelerate tumorigenesis in animals that are predisposed to cancer. Both T2/Onc concatemers and CAGGS-SB10 were crossed to *Arf*^{-/-} mice, animals deficient for the p53 pathway regulator and tumour suppressor p19Arf (ref. 13). Mice carrying either or both of the T2/Onc and CAGGS-SB10 transgenes were generated on the *Arf*^{-/-} background. The total number (n) and genotype of each group was as follows: *Arf*^{-/-};T2/Onc ($n = 54$), *Arf*^{-/-};CAGGS-SB10 ($n = 48$)

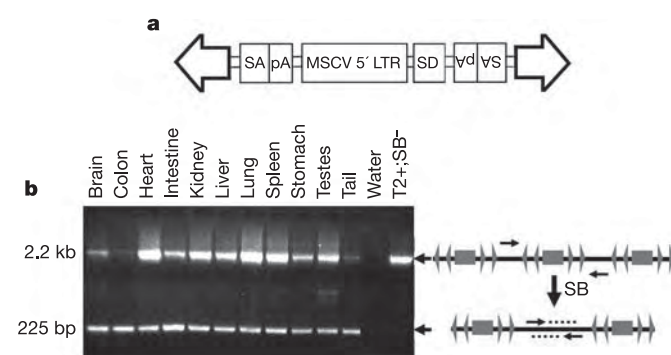


Figure 1 | Vector design and somatic transposition. **a**, The T2/Onc transposon contains elements to elicit transcriptional activation (MSCV 5' LTR and SD) and inactivation (SA and polyadenylation signals (pA)). **b**, A PCR excision assay shows somatic transposon excision within mice doubly transgenic for transposon and transposase. Primers detect a roughly 2.2-kb product (the size of the T2/Onc transposon) if a transposon has not mobilized from within the concatemer. If transposition and excision repair occur anywhere within the concatemer, a 225-base-pair PCR product is generated.

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and $Arf^{-/-}$;T2/Onc;CAGGS-SB10 ($n = 64$). A statistically significant decrease in time to morbidity in $Arf^{-/-}$ mice doubly transgenic for T2/Onc and CAGGS-SB10 compared with singly transgenic $Arf^{-/-}$ control animals ($P < 0.001$, by log-rank Mantel-Cox test) was observed (Fig. 2a). The tumour spectrum of $Arf^{-/-}$;T2/Onc;CAGGS-SB10 mice was similar to that previously reported in $Arf^{-/-}$ mice on the C57BL/6 genetic background¹⁴. Of 52 $Arf^{-/-}$;T2/Onc;CAGGS-SB10 animals analysed, 36 had soft tissue sarcomas or osteosarcomas (Fig. 2b, c, and data not shown). Lymphomas, malignant meningiomas, myeloid leukaemias and a pulmonary adenocarcinoma were also observed (data not shown). Comparing the two control groups $Arf^{-/-}$;CAGGS-SB10 and $Arf^{-/-}$;T2/Onc revealed no difference in time to morbidity ($P = 0.19$, log-rank Mantel-Cox test).

A Southern blot analysis of sarcoma genomic DNA from $Arf^{-/-}$;T2/Onc;CAGGS-SB10 mice detected the presence of multiple, clonal, T2/Onc transposon insertions (an average of five), whereas genomic DNA isolated from normal tissues from various locations of the same mice showed either no subclonal T2/Onc insertions or only one or two (Supplementary Fig. 1b, c). Genomic sequences immediately flanking somatically acquired transposon integration events in sarcomas from $Arf^{-/-}$ mice were amplified by linker-mediated polymerase chain reaction (PCR)¹⁵. A total of 1,053 distinct tumour-associated transposon integration events were cloned and sequenced from 28 tumours. In addition to cloning genomic integration events, we obtained the sequences immediately flanking the transgene concatemer in line 76. One end of this concatemer donor locus was cloned and mapped to chromosome 1 at 164,879,699 base pairs (data not shown). This was confirmed by fluorescence *in situ* hybridization (FISH, data not shown).

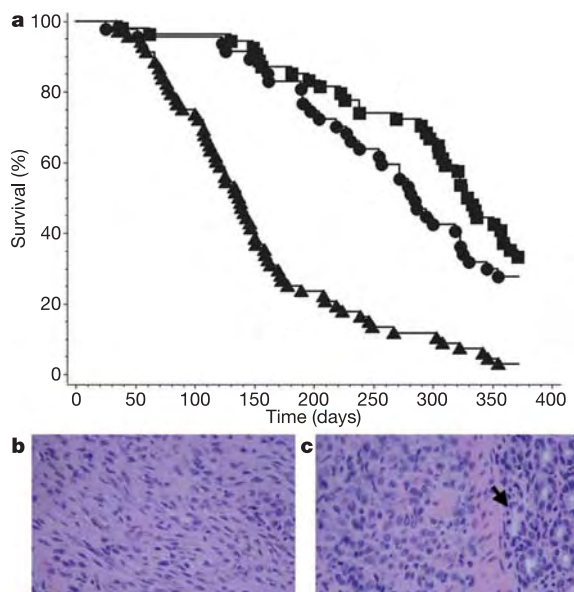


Figure 2 | $Arf^{-/-}$;T2/Onc;CAGGS-SB10 mice have a shorter tumour latency than singly transgenic controls. **a**, The Kaplan–Meier survival curve compares time to morbidity for $Arf^{-/-}$;T2/Onc;CAGGS-SB10 mice (triangles), $Arf^{-/-}$;CAGGS-SB10 mice (circles) and $Arf^{-/-}$;T2/Onc mice (squares). There is a statistically significant decrease in time to morbidity in $Arf^{-/-}$ mice doubly transgenic for T2/Onc and CAGGS-SB10 compared with singly transgenic $Arf^{-/-}$ controls ($P < 0.001$, log-rank Mantel–Cox test). This difference is evident when both distinct lines of T2/Onc transgenes are grouped or when they are assessed separately ($P < 0.001$ for both, data not shown). **b**, **c**, Examples of sarcomas from $Arf^{-/-}$;T2/Onc;CAGGS-SB10 mice: spindle cell tumour (undifferentiated sarcoma) found growing on the hindlimb (**b**); soft-tissue sarcoma infiltrating stomach glands (**c**; arrow).

In SB germline mutagenesis screens, 50–80% of transposons tend to reinsert within about 6 megabases (Mb) on either side of the donor concatemer^{16–18}. For somatic transposition in sarcomas, this ‘local hopping’ interval seems to be broadened, because only 23% of somatic integrations cloned from tumours from mice in line 76 occurred within the 40 Mb surrounding the concatemer (data not shown). The cloning of a large number of insertion sites also permitted mapping of the concatemer donor to chromosome 15 in line 68, confirmed by FISH (data not shown), and revealed a similar percentage of local hopping.

A genomic region mutated by the integration of T2/Onc in multiple different tumours, a common integration site (CIS), indicates possible selection for that event during tumorigenesis. On the basis of published Monte Carlo simulations, a CIS was defined as two integrations from two independent tumours in 13 kb, three or more integrations from three independent tumours in 269 kb, or two or more integration events from two independent tumours within the

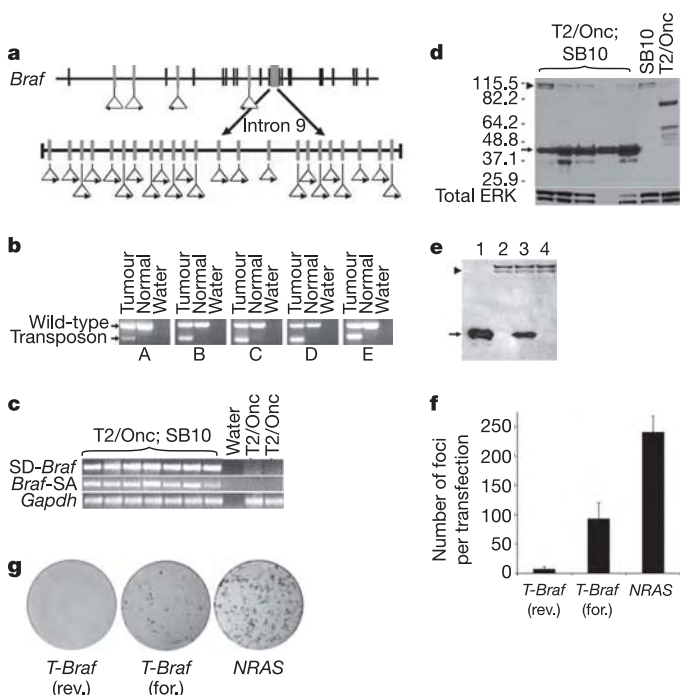


Figure 3 | Activation of *Brf* by T2/Onc insertion. **a**, Position and orientation of *Brf* T2/Onc insertions (grey). *Brf* exons are indicated by vertical black lines. The ninth intron is expanded to show detail of insertions. **b**, Three-primer PCR for ninth-intron *Brf* T2/Onc insertions (A–E) shows tumour specificity of insertions. **c**, RT-PCR reveals the presence of fusion transcripts, present in several T2/Onc;CAGGS-SB10 tumours harbouring ninth-intron insertions. The T2/Onc splice donor splices into the tenth exon of *Brf* (SD-*Brf*) and the *Brf* exon nine splice donor splices into the T2/Onc splice acceptor (*Brf*-SA). **d**, Western blot analysis detects a truncated C-terminal kinase domain of the BRAF protein (arrow) in sarcomas that harbour ninth-intron *Brf* integrations. Full-length BRAF protein is also detected (arrowhead). **e**, The SD-*Brf* transcript was amplified from tumours, cloned into an expression vector in the reverse (rev.) and forward (for.) orientations. Western analysis of 293T cells detects a truncated C-terminal kinase domain of the BRAF protein (arrow) and full-length BRAF protein (arrowhead): lane 1, tumour; lane 2, GFP-transfected; lane 3, forward; lane 4, reverse. **f**, **g**, Expression of truncated BRAF results in the formation of foci in NIH 3T3 cells. NRAS (G12V) is an acutely transforming oncogene for comparison. Error bars in **f** indicate standard deviation. **f**, Expression of truncated BRAF (T-*Brf* for) results in focus formation in NIH 3T3 cells. Transfection with the T-*Brf* rev control vector results in only baseline levels of focus formation. **g**, Representative plates showing focus formation.

same annotated gene^{19,20}. By these definitions, 54 CISs were identified by T2/Onc in *Arf*^{-/-} sarcomas (Table 1 and Supplementary Table 1). The local hopping phenomenon does increase the possibility of identifying CISs by random chance when they are linked to the concatemer donor locus. On the basis of published Monte Carlo simulations when insertions are distributed randomly and about 1,000 independent insertions are studied, 10 of our 54 CISs are predicted to occur simply by random chance¹⁹. As SB transposon integration favours sites linked to the donor locus, traditional Monte Carlo simulation (which assumes a completely random distribution of insertions throughout the genome) cannot accurately predict the number of false CISs occurring at loci linked to the transposon donor locus. The actual false-positive rate is therefore probably higher than this, because many CISs are linked to donor loci on chromosome 1 (for line 76) and chromosome 15 (for line 68) (Table 1 and Supplementary Table 1). However, it is likely that some linked CISs

are not identified merely by random chance. For example, several T2/Onc integrations in *Arf*^{-/-} tumours occurred in *Rabgap1l*, which is linked to the donor locus on chromosome 1 in line 76. *Rabgap1l* is a CIS identified in an accompanying paper²¹, providing additional evidence that *Rabgap1l* has a function in tumorigenesis. Despite local hopping, it seems that the entire genome is accessible to SB somatic mutagenesis, because T2/Onc integration events were cloned from all mouse chromosomes (data not shown) and CISs were also identified on chromosomes 2, 4, 6, 12, 13 and X. In addition, T2/Onc integrations were found near several CISs previously identified in leukaemias or lymphomas in retroviral mutagenesis screens^{22,23} (Supplementary Table 2).

The gene most frequently disrupted by T2/Onc was *Braf* (Table 1 and Supplementary Table 1). Integrations in or near *Braf* were cloned from 22 of 28 sarcomas and were found in tumours from mice transgenic for both T2/Onc concatemers, 68 and 76. Each sarcoma

Table 1 | Common integration sites in *Arf*^{-/-};T2/Onc;CAGGS-SB10 transgenic sarcomas

CIS name	Mouse chromosome	Approximate location (Mb)	Number of integrations	Number of independent tumours
Bai3	1	25.8	3	2
Dst	1	34.3	2	2
ENSMUST00000042986.3	1	56	2	2
Spag16	1	70	3	3
ENSMUSG00000042581	1	129	2	2
Daf1	1	130	3	2
NG-1-143	1	143	3	3
Uchl5	1	143.7	4	4
Rgs	1	144	10	6
B830045N13Rik	1	146.8	6	6
NG-1-147	1	147.2	4	4
NG-1-147b	1	147.7	3	3
Laminin	1	153	4	4
Creg	1	158	8	3
C80879	1	159	4	3
Rabgap1l	1	160	17	8
Tnfsf	1	161	4	4
Fmo	1	162	3	3
Prrx1	1	163	2	2
Dpt	1	164	4	4
ENSMUSG00000038473	1	170	2	2
Ptprt	2	161	2	2
Ptch2	4	115	2	2
NG-6-23	6	22	2	2
Cadps2	6	23.4	5	4
Braf	6	39	37	22
E330009J07Rik	6	40.3	4	4
ENSMUST00000071875.1	6	43	3	3
Cntnap2	6	46	3	3
Baiap1	6	94	2	2
ENSMUSETT00000078632	12	83	3	3
Adarb2	13	8.2	4	3
ENSMUSG00000039828	15	7.8	2	2
4933421G18Rik	15	8.1	2	2
ENSMUST00000082227.1	15	16	4	3
MGC92959	15	21.4	2	2
15-NG-22	15	22	3	3
ENSMUSG00000043556	15	26	3	3
ENSMUST00000075169.1	15	29	4	4
Catnd2	15	30.5	3	2
Sema5a	15	32	3	3
Coh	15	35.7	4	4
Rims2	15	39.3	2	2
2610028F08Rik	15	43.3	10	6
Trhr	15	43.9	25	11
Csmd3	15	47.8	14	8
ENSMUSETG00000033246	15	48.8	3	3
Rad21	15	52	6	4
LOC277923	15	55	2	2
BC026439	15	57.4	4	4
NG-15-69	15	69	6	5
ENSMUSETG00000029680	15	70	4	3
krt2	15	102	3	3
ENSMUST00000074972	X	137.7	2	2

See Supplementary Table 1 for additional details on integrations.

with *Braf* insertions had at least one within a TA dinucleotide in the ninth intron (Fig. 3a). All *Braf* ninth-intron insertions analysed seemed to be tumour-specific and absent from normal tissue from the same mouse (Fig. 3b); this is also true of other T2/Onc gene insertions studied (Supplementary Fig. 2).

All ninth-intron *Braf* integrations were directional with the MSCV LTR and splice donor oriented toward the tenth exon (Fig. 3a). This 'sense' orientation predicts that transcripts initiated from the MSCV LTR would splice into the tenth *Braf* exon, and this was confirmed by RT-PCR in seven sarcomas (Fig. 3c). This transcript could result in the expression of a truncated protein, translationally initiated in exon 10, containing the kinase domain of BRAF. An antibody against the carboxy-terminal fragment of BRAF detected a protein of the expected size (about 40 kDa) specifically in five sarcoma lysates that harbour intron 9 *Braf*-gene T2/Onc insertions (Fig. 3d). Moreover, an amino-terminal specific BRAF antiserum did not detect a truncated BRAF peptide (data not shown) despite the fact that a truncated *Braf* mRNA, generated by splicing from the *Braf* exon 9 splice donor into the splice acceptor upstream of the MSCV LTR sequences in T2/Onc, was detected (Fig. 3c).

Braf is a known oncogene, which has been shown to contain activating point mutations in 9% of human sarcoma cell lines and 0.5–5% of primary human sarcomas^{24,25}. This provides a proof of principle that SB somatic mutagenesis identifies genes associated with, and clinically relevant to, specific human cancers. The truncated BRAF protein expressed in sarcomas with T2/Onc integrations in the ninth intron of *Braf* contains only the kinase domain, lacks N-terminal negative regulatory elements of the protein and is capable of morphological transformation of NIH 3T3 cells (Fig. 3e–g). Previous work has shown the oncogenic potential of a truncated kinase domain of the closely related *Craf* (ref. 26). On the basis of these results, it seems that *Braf* is capable of collaborating with *Arf* loss to elicit sarcoma development.

We have shown that *Sleeping Beauty* can be used for somatic-cell insertional mutagenesis in the mouse for the identification of cancer genes in solid tumours. In the future, T2/Onc mobilization combined with tissue-specific loss of a tumour suppressor might permit the identification of tumour-predisposing genes for any tissue type. In an accompanying paper²¹ it is shown that the improved SB transposase, SB11 (ref. 27), expressed from the *Rosa26* locus can cause the efficient somatic mobilization of a T2/Onc-like transposon and induce tumour formation in the absence of a cancer-predisposed genetic background. The differences in the ability of T2/Onc mobilization by CAGGS-SB10 and *Rosa26*-SB11 to initiate and promote tumour formation might be due to differences in activity of SB10 and SB11, differences in levels of protein expression or differences in spatial or temporal expression of the transposase. The use of a conditionally expressed SB transposase may improve future studies by allowing control of its spatial and temporal expression. In addition, new transposon vector designs may further enhance the utility of the system.

METHODS

Vector construction. The T2/Onc vector contains the MSCV 5' LTR from the MSCVneo vector (Clontech). The splice donor is from exon 1 of the mouse *Foxf2* gene. One splice acceptor is derived from exon 2 of the mouse *engrailed-2* gene and the other from the carp β -actin gene. Each is followed by the bi-directional SV40 poly(A).

Mice. Transgenic lines of T2/Onc were generated on the FVB/N genetic background. Southern blot analysis was performed on genomic DNA from a tail biopsy, and two lines (nos 68 and 76) with high copy numbers (about 25 copies) and a lack of transgene methylation were chosen for further analysis. The copy number of T2/Onc elements within the concatemer was estimated by comparison of T2/Onc signal intensity from transgenic genomic DNA to known amounts of T2/Onc plasmid DNA by Southern analysis. Methylation status was investigated by Southern analysis after digestion with a methylation-sensitive restriction enzyme. It was proposed that methylation of the transposon transgene might silence the activity of the MSCV LTR after transposition to new

sites in the genome. FISH and spectral karyotyping analysis were used to map concatemer 68 to chromosome 15 and concatemer 76 to chromosome 1. To generate the *Arf*^{+/−} cohort, *Arf*^{+/−};CAGGS-SB10 and *Arf*^{+/−};T2/Onc mice were first generated by crossing *Arf*^{+/−} mice with CAGGS-SB10 or T2/Onc mice, respectively. *Arf*^{+/−};CAGGS-SB10 mice were intercrossed to generate *Arf*^{+/−};CAGGS-SB10 mice. *Arf*^{+/−};T2/Onc mice were also intercrossed to generate *Arf*^{+/−};T2/Onc mice. *Arf*^{+/−};CAGGS-SB10 mice and *Arf*^{+/−};T2/Onc mice were crossed to generate *Arf*^{+/−};CAGGS-SB10;T2/Onc, *Arf*^{+/−};T2/Onc and *Arf*^{+/−};CAGGS-SB10 mice.

PCR primers. Sequences of PCR primers are given in Supplementary Information.

Histopathology. Tissues were fixed overnight in 10% formalin at 4 °C, stored in 70% ethanol, embedded in paraffin, sectioned and stained with haematoxylin and eosin.

Linker-mediated PCR and 'shot-gun cloning'. Linkers used to clone insertions were described previously¹⁵. Genomic DNA was digested with *Nla*III and *Xho*I (for cloning from the right side of T2/Onc) or *Bfa*I and *Bam*HI (for cloning from the left side of T2/Onc) and ligated to the linker. Primary PCR was used to amplify sequences flanking the IR/DR. Primary PCR products were diluted 1:50 and used in a secondary PCR. Secondary PCR products were ligated to pGEM-T Easy (Promega) and electroporated into DH10B Electromax-competent cells (Invitrogen). Library plating, colony picking and sequencing with the SP6 primer in 96-well format was performed by Agencourt Biosciences. Automated database searches of T2/Onc integration sites were performed as described previously by K. Akagi²². The closest gene to each integration (within 100 kb on either side of the integration) was determined with a combination of the UCSC (<http://www.genome.ucsc.edu>) and Ensembl (<http://www.ensembl.org>) mouse whole-genome annotations (NCBI m33 build).

***Braf* three-primer PCR.** Genomic DNA (500 ng) was used in a PCR with three separate primers. One transposon-specific primer was used for all three-primer PCRs together with specific primers to detect the wild-type locus of each cloned insertion event.

***Braf* RT-PCR.** Total RNA was isolated from tumour tissues by TRIzol (Invitrogen), contaminating DNA was removed by treatment with DNase (Invitrogen) and RT-PCR was performed with 500 ng of RNA with the RobusT I RT-PCR kit (Finnzymes, MJ Research). A splice-donor (SD)-specific primer and a *Braf* tenth-exon-specific primer were used. A *Braf* exon-seven-specific primer and a Carp β -actin splice-acceptor (SA)-specific primer were used. The resulting products were sequenced to verify the fidelity of each splicing event.

BRAF western blot analysis. Protein lysates were prepared by homogenizing tissue in IPWB lysis buffer (50 mM Tris-HCl pH 7.4, 14.6 mg ml^{−1} NaCl, 2 mM EDTA, 2.1 mg ml^{−1} NaF, 1% Nonidet P40, 1 mM NaVO₄ and 1 mM Na₂PO₄, containing protease inhibitors (Roche)) or by following the manufacturer's protocols for protein isolation from the organic phase of TRIzol. Samples (about 30 μ g) were subjected to electrophoresis on a 4–12% Bis-Tris gel and transferred to nitrocellulose (BioRad) with the NuPAGE system (Invitrogen). Blots were probed with a primary antibody specific to the carboxy terminus of BRAF (Santa Cruz Biotechnology). An antibody specific for Erk-1 (Santa Cruz Biotechnology) was used as a loading control.

Cloning of truncated *Braf* and NIH 3T3 transformation assay. The cDNA of the truncated C-terminal fusion transcript of *Braf* generated in tumours (T2/Onc SD exons 10–19 of *Braf*) was amplified with the RobusT I RT-PCR kit. The amplified product was subcloned into pCR 2.1-TOPO (Invitrogen), excised with *Eco*RI and ligated in the forward and reverse orientations into an *Eco*RI site of a CAGGS vector. These plasmids and a CAGGS plasmid expressing the activated human *NRAS* oncogene (G12V) (C.M.C. and D.A.L., unpublished observations) were each transfected in duplicate into NIH 3T3 cells with the Superfect Transfection Reagent (Qiagen). Cells were cultured in DMEM medium with 10% FBS, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 55 μ M 2-mercaptoethanol and 10 μ g ml^{−1} gentamycin, split into two 100-mm plates 2 days after transfection, cultured for 10 days and stained with methylene blue.

Received 4 February; accepted 27 April 2005.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements We thank M. Roussel for providing *Arf*^{−/−} mice; P. Woll, S. Rosenthal, R. Wang, P. Lobitz, J. Zinggeler and M. Davies for technical assistance; P. Hackett, S. McIvor, S. Ekker, P. Marker and members of the Largaespada laboratory for critical reading of the manuscript; N. Kirchhof for performing the pathology; T. L. Bergemann for providing assistance with statistical analysis; and K. Akagi for performing automated sequence analysis and BLAT searches. T2/Onc transgenic mice were generated by the University of Minnesota Mouse Genetics Laboratory. This work was supported by the Arnold and Mabel Beckman Foundation, the National Institute of Drug Abuse and the National Cancer Institute.

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Gli3 and *Plzf* cooperate in proximal limb patterning at early stages of limb development

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The vertebrate limb initially develops as a bud of mesenchymal cells that subsequently aggregate in a proximal to distal (P–D) sequence to give rise to cartilage condensations that prefigure all limb skeletal components¹. Of the three cardinal limb axes, the mechanisms that lead to establishment and patterning of skeletal elements along the P–D axis are the least understood. Here we identify a genetic interaction between *Gli3* (GLI-Kruppel family member 3) and *Plzf* (promyelocytic leukaemia zinc finger, also known as *Zbtb16* and *Zfp145*), which is required specifically at very early stages of limb development for all proximal cartilage condensations in the hindlimb (femur, tibia, fibula). Notably, distal condensations comprising the foot are relatively unperturbed in *Gli3*^{−/−};*Plzf*^{−/−} mouse embryos. We demonstrate that the cooperative activity of *Gli3* and *Plzf* establishes the correct temporal and spatial distribution of chondrocyte progenitors in the proximal limb-bud independently of known P–D patterning markers and overall limb-bud size. Moreover, the limb defects in *Gli3*^{−/−};*Plzf*^{−/−} embryos correlate with the transient death of a specific subset of proximal mesenchymal cells that express bone morphogenetic protein receptor, type 1B (*Bmpr1b*) at the onset of limb development. These findings suggest that the development of proximal and distal skeletal elements is distinctly regulated early during limb-bud formation. The initial division of the vertebrate limb into two distinct molecular domains is consistent with fossil evidence indicating that the upper and lower extremities of the limb have different evolutionary origins².

The basic adult pattern of the vertebrate limb skeleton derives from a cartilaginous model: a single proximal long bone within the stylopod segment (humerus; femur), followed by two long bones within the zeugopod segment (radius, ulna; tibia, fibula) and then the distal autopod segment comprising of wrist or ankle, and digits. This initial cartilage pattern is preceded by *Sox9* expression, which marks the chondrocyte precursors of all three segments^{3,4}. Several factors, such as *Hox* genes or fibroblast growth factor (*Fgf*) genes, elaborate this initial cartilage pattern either by driving proliferation of specific limb segments along the P–D axis once they are established^{5,6} (*Hox*), or by ensuring that there are adequate numbers of mesenchymal cells in the limb-bud to form skeletal elements of the correct size⁷ (*Fgf*). However, these mouse mutants display a relatively unperturbed initial cartilage pattern, as revealed by alcian-blue staining^{5,6} or *Sox9* expression, which is initially evident in all three P–D segments despite drastically reduced limb-bud size⁷. For example, *Hoxd11*^{−/−};*Hoxa11*^{−/−} mice exhibit a specific loss of the forelimb zeugopod element: the initial cartilage elements comprising the radius and ulna form, but subsequent development is perturbed^{5,6}. On the other hand, mutations in genes that affect chondrogenesis, such as *Sox9*, affect the formation of all chondrogenic elements⁴. Therefore, the mechanisms that regulate early events

underlying P–D limb patterning are largely unknown.

Here we identified a cooperative role of two transcription factors, *Gli3* and *Plzf*, in the regulation of proximal limb development. *Gli3*^{−/−};*Plzf*^{−/−} double-knockout embryos (see Methods) display a phenotype distinct from the anterior to posterior limb-patterning defects observed in either single mutant^{8–10} (Supplementary Fig. 1). All proximal skeletal structures of *Gli3*^{−/−};*Plzf*^{−/−} hindlimbs are severely affected, whereas the distal autopod skeleton is relatively unperturbed (Fig. 1 and Supplementary Fig. 1). Severely affected *Gli3*^{−/−};*Plzf*^{−/−} hindlimbs have only a single piece of proximal

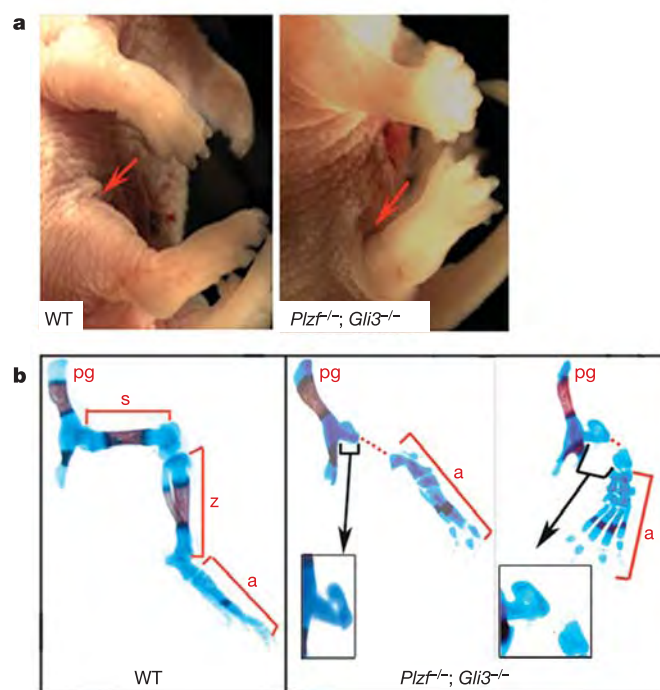


Figure 1 | *Plzf* and *Gli3* regulate proximal skeletal patterning. **a**, External morphology of E16.5 wild-type (WT) and *Plzf*^{−/−};*Gli3*^{−/−} embryos. Red arrow indicates where hindlimb meets the body wall. **b**, Skeleton of E16.5 hindlimbs demonstrating severe stylopod and zeugopod defects in *Gli3*^{−/−};*Plzf*^{−/−} embryos. Two *Gli3*^{−/−};*Plzf*^{−/−} examples illustrate the most severe phenotype (left) with a single proximal cartilage element and the milder phenotype (right) with two proximal cartilage pieces. In both cases, the proximal element attached to the pelvic girdle (pg) resembles a malformed knee joint. Dashed red line denotes a gap between skeletal elements. The pelvic girdle, which is not derived from the hindlimb bud, appears normal. a, autopod; s, stylopod; z, zeugopod.

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cartilage (1 embryo of 12 analysed) that morphologically resembles a malformed knee joint (Fig. 1b). Other representative *Gli3*^{-/-};*Plzf*^{-/-} hindlimbs have variable numbers (2–5) of small pieces of proximal cartilage, which lack the morphology of stylopod and zeugopod elements (Fig. 1b and Supplementary Fig. 1; 11/12). These proximal structures are small, amorphous pieces of cartilage, with the notable exception of a consistent structure resembling an apparent knee joint that is often fused to the pelvic girdle. Despite these proximal defects, the autopod is present and similar to *Gli3*^{-/-} embryos. However, digit number is often reduced to four digits, and there is partial rescue in the overall shape or size of tarsal elements and phalange 1 compared to *Gli3*^{-/-} hindlimbs (Supplementary Fig. 1 and Fig. 1a). Frequently, the calcaneus, an ankle bone, is elongated proximally in *Gli3*^{-/-};*Plzf*^{-/-} hindlimbs (Supplementary Fig. 1; 8/12). There are no discernible P–D skeletal defects in *Gli3*^{-/-};*Plzf*^{-/-} forelimbs (data not shown); however, the embryos die before birth, as do *Gli3*^{-/-} single mutants¹⁰. We therefore conclude that *Gli3* and *Plzf* specifically cooperate in the development of the hindlimb stylopod and zeugopod.

We determined when these skeletal defects become apparent by examining the earliest cartilage pattern by alcian-blue staining at embryonic day (E) 12.5. *Gli3*^{-/-} or *Plzf*^{-/-} single-mutant hindlimbs show a wild-type cartilage pattern, except for extra digit condensations in *Gli3*^{-/-} embryos (Supplementary Fig. 1). In contrast, although *Gli3*^{-/-};*Plzf*^{-/-} hindlimbs show a relatively normal autopod, morphologically discernible stylopod and zeugopod elements are not apparent (Fig. 2, top panels). Instead, in all E12.5 *Gli3*^{-/-};*Plzf*^{-/-} hindlimbs, a single ball of proximal cartilage is present that does not possess the elongated long-bone morphology

characteristic of either the stylopod or zeugopod. The cartilage ball is initially located at the approximate position of the knee joint, coincident with proximal joint formation in wild-type limbs, and at E16.5 an apparent knee joint is present in all *Gli3*^{-/-};*Plzf*^{-/-} hindlimbs (Fig. 1b), although it often fuses to the pelvic girdle. This shift in relative position may result from the absence of proximal long-bone cartilage elements during limb outgrowth. Therefore, we examined expression of joint-specific markers. In E12.5 wild-type hindlimbs, joint markers are expressed in a horseshoe-like stripe where the developing knee joint forms to delineate the area between stylopod and zeugopod (Fig. 2, middle panels), and these cells co-express the cartilage marker collagen, type IIa (*Col2a*). The *Gli3*^{-/-};*Plzf*^{-/-} limbs also express the joint-specific markers *Gdf5*, *Wnt9a* and *Fgf18*, but these markers entirely encompass the single *Col2a*-positive and the alcian-blue-stained proximal ball of cells (Fig. 2, middle panels; data not shown). By E14.5, the proximal cartilage ball in *Gli3*^{-/-};*Plzf*^{-/-} limbs frequently separates into one or more pieces, each of which retains expression of joint markers and *Col2a*, whereas joint markers are expressed between the *Col2a* domains in wild-type limbs (Fig. 2, bottom panels; Supplementary Fig. 2; data not shown). Joint precursors and other mesenchymal derivatives, such as tendon, are not initially expanded in *Gli3*^{-/-};*Plzf*^{-/-} limbs at E11.5 (Supplementary Fig. 2 and data not shown) and are expressed in a region similar to that observed in wild-type limbs, indicating that proximal skeletal elements are not misdirected towards a joint or tendon fate. We therefore conclude that formation of the stylopod and zeugopod, but not the proximal joint, is specifically disrupted at very early stages of limb development in *Gli3*^{-/-};*Plzf*^{-/-} embryos.

Histology of *Gli3*^{-/-};*Plzf*^{-/-} limbs showed no evidence of mesenchymal condensation other than the single cartilage ball that expresses joint markers and the autopod at E12.5 (Fig. 3a), and no condensations between separated proximal cartilage pieces at E14.5 (data not shown). Even at the time when wild-type stylopod/zeugopod condensations are first initiated, there is no

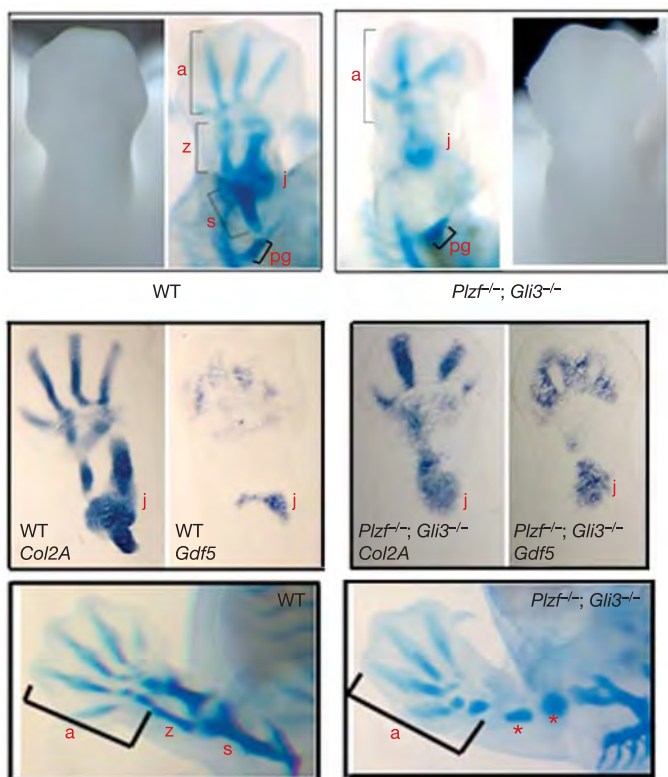


Figure 2 | *Plzf* and *Gli3* regulate molecular and morphological features of the proximal stylopod and zeugopod. Upper panels, E12.5 wild-type and *Gli3*^{-/-};*Plzf*^{-/-} hindlimbs photographed and then stained with alcian-blue to reveal the skeleton. Middle panels, *in situ* hybridizations on hindlimb sections at E12.5 for markers of cartilage (*Col2a*) and joint (*Gdf5*). Lower panels, E14.5 hindlimbs stained with alcian-blue. Asterisks indicate the cartilage pieces that would also express joint markers. j, joint.

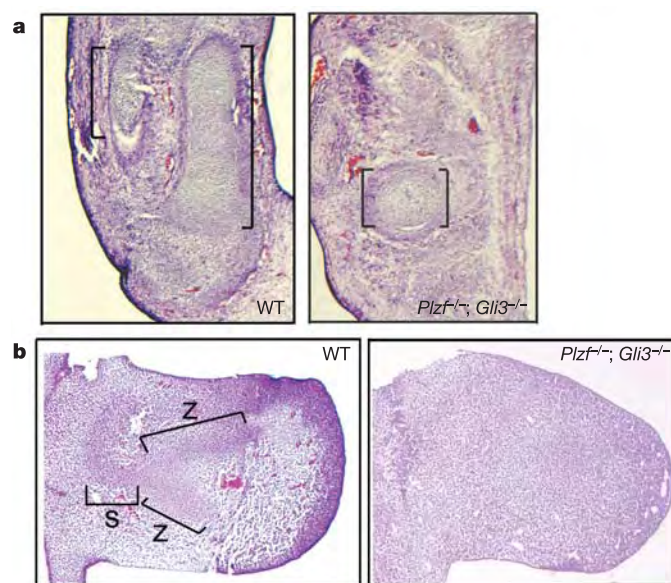


Figure 3 | *Plzf* and *Gli3* are required for proximal limb condensations. a, b, Histology of wild-type and *Plzf*^{-/-};*Gli3*^{-/-} hindlimb sections at E12.5 (a) and E11.5 (b) stained with haematoxylin and eosin. a, E12.5 *Gli3*^{-/-};*Plzf*^{-/-} limbs show only a single ball of mesenchymal condensation within the proximal region, which lacks the long-bone morphology of wild-type proximal skeletal elements indicated by brackets. b, E11.5 *Gli3*^{-/-};*Plzf*^{-/-} limbs have no discernible chondrogenic mesenchymal condensations.

evidence of proximal condensations in *Gli3*^{-/-};*Plzf*^{-/-} limbs (Fig. 3b), although a half-day later a digit condensation is formed (Supplementary Fig. 3). This raises the question of whether *Plzf* and *Gli3* act at or before the condensation stage to regulate proximal skeletal development.

We therefore examined the earliest-known marker for cartilage differentiation, *Sox9* (refs 3, 4), which is expressed one day before initiation of limb cartilage condensations. Remarkably, at a time when *Sox9* expression is normally initiated in wild-type embryos, there is no *Sox9* expression in *Gli3*^{-/-};*Plzf*^{-/-} hindlimbs (Fig. 4a). At E11.5, *Sox9* is expressed normally in the *Gli3*^{-/-};*Plzf*^{-/-} autopod, consistent with its formation in mutant hindlimbs. However, in proximal regions there is only a small clump of *Sox9*-positive precursors (Fig. 4a), which may correspond to the later proximal cartilage piece revealed by histology, alcian-blue staining and joint marker expression at E12.5 (Figs 2 and 3). These results place *Gli3* and *Plzf* function before cartilage differentiation.

Despite proximal patterning defects, *Gli3*^{-/-};*Plzf*^{-/-} limb-bud size is indistinguishable from that of wild types before E11.5, and

only slightly smaller at E12.5 (Figs 2 and 3). We examined whether loss of proximal skeletal elements correlated with changes in expression of markers of limb growth and patterning (see Methods). *Fgf4*, *Fgf8*, *Gremlin*, *Hoxd9*, *Hoxd10* and *Hoxd11* all showed anteriorized expression similar to either *Gli3* or *Plzf* single mutants^{8-10,11} (Supplementary Fig. 4). Although specific markers for P-D elements are unknown, *Meis1* and *Meis2* expression marks the proximal limb region¹². These genes and other P-D markers such as *Hoxa9*, *Hoxa10* and *Hoxa11* are normally expressed in *Gli3*^{-/-};*Plzf*^{-/-} limbs (Fig. 4b and Supplementary Fig. 5). Thus, combined *Gli3* and *Plzf* function is required independently from proposed P-D markers.

As there are no other known markers for limb mesenchyme specification before *Sox9* expression, we investigated whether distinct cellular populations were affected by assessing cell proliferation and death (see Methods). Cell proliferation is not altered in *Gli3*^{-/-};*Plzf*^{-/-} limbs compared to the wild type at E10.5 (Fig. 4e). Strikingly, however, at E10.5 there is a small domain of cell death restricted to the most proximal region of *Gli3*^{-/-};*Plzf*^{-/-}

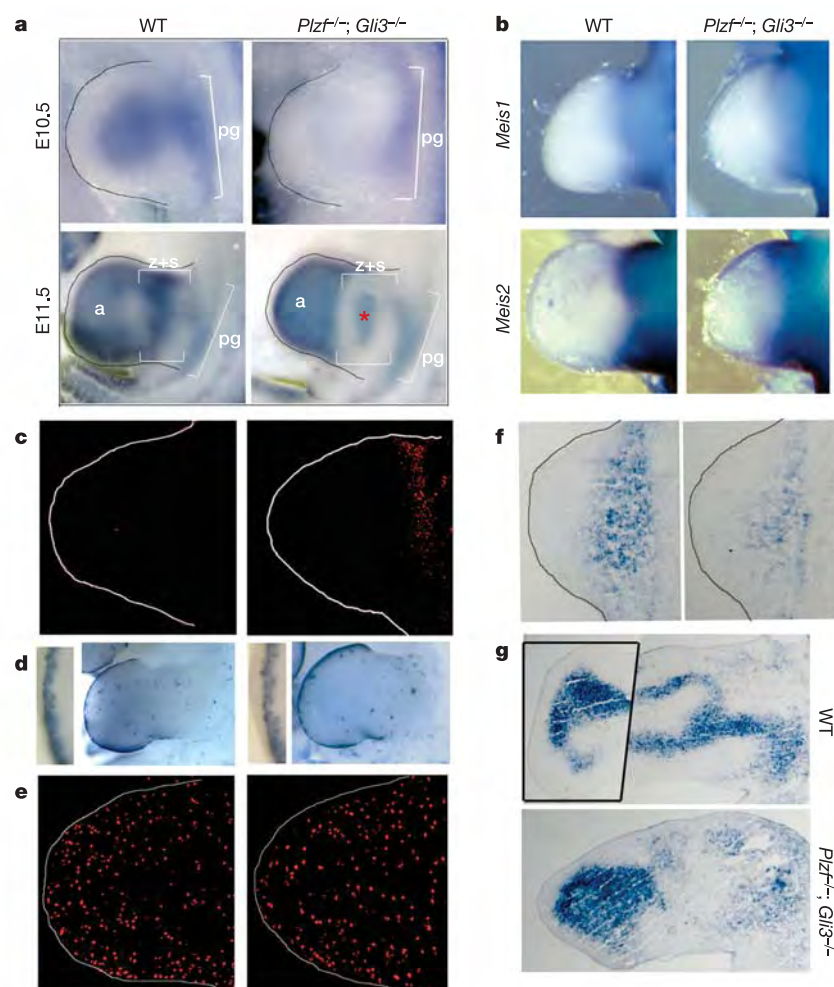


Figure 4 | Cellular consequences of *Plzf* and *Gli3* loss of function. **a**, *Sox9* messenger RNA expression in hindlimbs by whole-mount *in situ* hybridization. Red asterisk denotes small clump of *Sox9*-positive cells, which may relate to variable cartilage fragments in older *Gli3*^{-/-};*Plzf*^{-/-} hindlimbs. **b**, Expression of P-D markers as indicated at E10.5. **c**, **d**, TUNEL staining on E10.5 sections (**c**) and E11.5 whole-mounts (**d**). Note transient proximal cell death domain in E10.5 *Gli3*^{-/-};*Plzf*^{-/-} hindlimbs in **c**. Insets in **d**, magnification (×5) of apoptotic cells in the apical ectodermal ridge. **e**, Cell proliferation assayed by anti-phosphorylated histone H3 antibody at E10.5. No difference in cell proliferation was detected by quantifying

percentage of mitotic cells to total nuclei in distal (wild type: 19%; *Gli3*^{-/-};*Plzf*^{-/-}: 16.5%) and proximal (wild type: 15%; *Gli3*^{-/-};*Plzf*^{-/-}: 18%) limb regions (average of 500 cells counted in each region). The limb-bud is highlighted by black or white outlining. **f**, *Bmpr1b* expression at E10.5 by section *in situ* hybridization. Note that *Bmpr1b*-positive cells are located similarly to the dying cells in *Gli3*^{-/-};*Plzf*^{-/-} hindlimbs (**c**) and this population is reduced in *Gli3*^{-/-};*Plzf*^{-/-} limbs. **g**, *Bmpr1b* expression at E11.5. Box in WT indicates overlay of the autopod from a serial section of the same hindlimb. Distal is to the left, posterior to the bottom.

hindlimb buds close to the body wall (Fig. 4c; $n = 2$). This cell death is transient, and at E11.5 no differences are detected between $Gli3^{-/-};Plzf^{-/-}$ and wild-type limbs (Fig. 4d and data not shown for limb sections; $n = 4$). Importantly, the dying cells in $Gli3^{-/-};Plzf^{-/-}$ hindlimbs reflect only a small fraction of proximal mesenchyme marked by *Meis* or *Sox9* expression in wild-type limbs (Fig. 4b and Fig. 4c), consistent with the normal size of $Gli3^{-/-};Plzf^{-/-}$ hindlimbs (Fig. 2). Taken together, these results suggest that cells undergoing apoptosis in $Gli3^{-/-};Plzf^{-/-}$ hindlimbs may reflect small cell populations required to correctly establish or organize the initial stylopod/zeugopod condensations, rather than the mesenchymal cells that contribute to their subsequent expansion. The fact that both *Gli3* and *Plzf* are initially expressed throughout the entire early limb-bud^{8,10,13} indicates that these two transcription factors cannot, by themselves, be used as markers of these cells.

To further investigate the proximally restricted dying cells in $Gli3^{-/-};Plzf^{-/-}$ limbs we examined expression of *Bmpr1b*, which marks cellular populations that prefigure the future limb cartilage primordium^{14–16}. We identified a small group of previously unrecognized *Bmpr1b*-positive cells in the most proximal region of the E10.5 wild-type hindlimb (Fig. 4f), whereas these cells lie within the forelimb future stylopod region (Supplementary Fig. 6 and see ref. 13). Strikingly, in $Gli3^{-/-};Plzf^{-/-}$ hindlimbs, *Bmpr1b*-positive cells overlap the apoptotic region and this population is greatly reduced (Fig. 4f). At E11.5, *Bmpr1b* expression clearly marks wild-type hindlimb stylopod, zeugopod and autopod primordia before histological condensation, whereas in $Gli3^{-/-};Plzf^{-/-}$ hindlimbs, *Bmpr1b* is specifically absent in the stylopod/zeugopod region (Fig. 4g). Thus, a small subset of cells marked by *Bmpr1b* expression is critically affected at the earliest stages of limb development in $Gli3^{-/-};Plzf^{-/-}$ embryos and correlates with subsequent loss of proximal skeletal elements (Fig. 3a). This suggests *Gli3* and *Plzf* are required to specify a subset of mesenchymal cells needed for the subsequent recruitment and formation of proximal condensations.

Our results demonstrate that *Gli3* and *Plzf* are specifically required for formation of stylopod and zeugopod elements at very early stages of limb development, before the initiation of cartilage condensations. These findings uncover a novel step regulated by *Gli3* and *Plzf* that is independent of known P–D markers and is required for establishing the correct timing and location of chondrocyte precursors specifically within proximal regions of the developing limb. Therefore, *Gli3* and *Plzf* cooperatively promote the proximal limb fate. However, as both *Gli3* and *Plzf* are broadly present throughout the developing limb without P–D bias in expression or activity, they cannot serve as markers of proximal limb identity^{8,10,13,17}. We have also identified a requirement for *Gli3* function that appears to be independent of *Shh* signalling, because the defect is markedly different from *Shh*^{-/-} limbs or from *Shh*^{-/-};*Gli3*^{-/-} limbs in which all skeletal elements are rescued^{18–19}.

Our studies provide a mouse model that directly addresses several unresolved questions in P–D limb patterning. First, our findings address whether P–D cartilage elements form via a branching mechanism^{20,21}. The fact that distal structures form in $Gli3^{-/-};Plzf^{-/-}$ limbs while proximal condensations are severely affected argues that P–D development relies on formation of individual condensations that are independently regulated. Second, $Gli3^{-/-};Plzf^{-/-}$ limbs provide insight into how individual P–D cartilage condensations may be initiated. Previous studies suggest that the initial formation of condensations is dependent on the number of limb mesenchymal cells^{7,22}. Importantly, despite gross alterations in proximal limb patterning, $Gli3^{-/-};Plzf^{-/-}$ limb-bud size and mesenchyme cell number is normal before E11.5, and only slightly reduced at E12.5. Moreover, only a single proximal condensation that expresses joint markers is formed when the stylopod/zeugopod/joint cartilage template is evident in wild-type limbs. This raises the possibility that a separate population of joint precursors exists, or that a

common joint/cartilage progenitor retains the ability to differentiate into proximal joints, but not into long bones, in $Gli3^{-/-};Plzf^{-/-}$ limbs. Intriguingly, we identified a small population of *Gli3*- and *Plzf*-dependent *Bmpr1b*-positive cells present at the onset of limb development that may be required to establish initial cartilage condensations correctly. Therefore, although the overall number of mesenchymal cells is important for expansion of cartilage elements, *Bmpr1b*-positive cells may serve a distinct function necessary for formation of ‘aggregation or organisation centres’ to recruit cells from the surrounding mesenchyme into initial condensations, thus prefiguring the limb skeleton^{15,20}.

Our findings genetically demonstrate that the formation of proximal and distal skeletal patterning is differentially regulated very early in limb development. The finding that the autopod forms in the absence of the proximal elements, although overall limb size and P–D patterning is normal, is inconsistent with the idea of progressive specification raised by the Progress Zone Model²³. In contrast, our findings are consistent with fate-mapping studies which propose that distinct P–D progenitor populations are specified early in an independent, non-progressive manner (Early Specification Model)²⁴. Our results therefore extend and provide a genetic framework to test ideas proposed by these models.

METHODS

Animals. *Gli3*- and *Plzf*-deficient mouse embryos were obtained by intercrossing mice carrying the *Gli3*^{X⁺J} allele¹⁰ with mice carrying the targeted inactivation of *Plzf* (ref. 8) and genotyped as described^{15,25}.

Staining and whole-mount *in situ* hybridization. Alcian-blue and alizarin-red staining of cartilage and bone, and whole-mount *in situ* hybridization analyses were performed as described⁸.

TUNEL and immunofluorescence assays. Apoptotic cells were detected *in situ* on frozen sections or in whole mount²⁶ by incorporating fluorescein-dUTP or Dig-11-dUTP, respectively, into fragmented DNA using terminal transferase according to the manufacturer's instructions (Roche Diagnostics). To detect cells in mitosis, a rabbit anti-phosphorylated histone H3 antibody (Upstate Biotechnology) and Cy-3 conjugated goat anti-rabbit IgG antibody (Jackson Lab) were used. Total nuclei were detected by DAPI staining (Jackson Lab) during the last wash of the immunofluorescence. Nonspecific antibody binding was blocked by incubating limb sections for 1 h at room temperature (25 °C) in PBT (PBS with 0.1% Tween-20) with 1% heat-inactivated goat serum (HIGS). All staining reactions were performed in PBT with 1% HIGS for 1 h at room temperature, except for incubation with primary antibody overnight at 4 °C in a humidified chamber.

Received 12 January; accepted 6 May 2005.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements We thank our laboratory staff, in particular S. Weatherbee and I. Zohn, for discussions; D. Ruggero for suggestions and support; and Y. Yang for discussions on joint formation. We thank Y. Yang, C. Tabin, D. Kingsley, A. Boulet, M. Capecchi and B. de Crombrughe for *in situ* hybridization probes. This work was supported by the NIH (L.N.) and by a MSKCC Cancer Center Support grant. L.N. is an Investigator of the Howard Hughes Medical Institute.

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LETTERS

An *Arabidopsis* *hAT*-like transposase is essential for plant development

Paul Bundock^{1†} & Paul Hooykaas¹

A significant proportion of the genomes of higher plants and vertebrates consists of transposable elements and their derivatives. Autonomous DNA type transposons encode a transposase that enables them to mobilize to a new chromosomal position in the host genome by a cut-and-paste mechanism. As this is potentially mutagenic, the host limits transposition through epigenetic gene silencing and heterochromatin formation. Here we show that a transposase from *Arabidopsis thaliana* that we named *DAYSLEEPER* is essential for normal plant growth; it shares several characteristics with the *hAT* (*hobo*, *Activator*, *Tam3*) family of transposases¹. *DAYSLEEPER* was isolated as a factor binding to a motif (Kubox1) present in the upstream region of the *Arabidopsis* DNA repair gene *Ku70* (refs 2, 3). This motif is also present in the upstream regions of many other plant genes. Plants lacking *DAYSLEEPER* or strongly overexpressing this gene do not develop in a normal manner. Furthermore, *DAYSLEEPER* overexpression results in the altered expression of many genes. Our data indicate that transposase-like genes can be essential for plant development and can also regulate global gene expression. Thus, transposases can become domesticated by the host to fulfil important cellular functions.

A one-hybrid screen in yeast was performed to identify proteins from *Arabidopsis* that bind to the upstream region of the *Arabidopsis* DNA repair gene, *Ku70* (refs 2, 3). Essentially, a 1-kilobase pair (1-kbp) fragment of the *Arabidopsis* *Ku70* upstream region was cloned in front of the yeast *HIS3* gene. This was then integrated into the yeast genome, and a library of *Arabidopsis* complementary DNAs fused to the *GAL4* activation domain was introduced. Yeast colonies growing on medium lacking histidine were analysed further. We identified several cDNAs of different lengths from the gene At3g42170 (GenBank NM114084). This gene, which we named *DAYSLEEPER*, encodes a protein with the conserved domains from the family of *hAT* transposases that includes *hobo* from *Drosophila melanogaster*, *Activator* (*Ac*) from maize, and *Tam3* from snapdragon^{1,4} (see Supplementary Fig. S1) but lacks several amino acids known to be essential for *Ac* transposition⁵. In *Arabidopsis*, 246 *hAT*-like elements have been identified⁶. Active *hAT* transposons are delimited by an 8 bp duplication of the insertion site and short terminal inverted repeats (TIRs). *hAT* transposons lacking these features are considered fossil elements that are unable to transpose and these are often transcriptionally silent. The *DAYSLEEPER* locus also lacks 8 bp duplications and TIRs. However, *DAYSLEEPER* expression can be detected, several expressed sequence tags are available, and furthermore expression also seems to be under the control of factors determining the circadian rhythm (<http://www.arabidopsis.org>). This suggested that *DAYSLEEPER* may have acquired an alternative cellular function.

Using shorter fragments of the *Ku70* upstream region in a one-hybrid assay, we localized the *DAYSLEEPER* binding site to a 229 bp

fragment located 60 bp before the *Ku70* transcription start (see Supplementary Fig. S2). This binding was also confirmed in a gel retardation assay. This fragment contains three imperfect repeats of the 25 bp sequence Kubox1 (see Supplementary Fig. S3). We were unable to find any homology between the Kubox1 motif and known *hAT* transposon TIRs¹ or their subterminal regions, which also often contain transposase binding sites⁷. In order to establish that Kubox1 constituted the *DAYSLEEPER* binding site, double stranded multimers of Kubox1 were used in a gel retardation assay. *DAYSLEEPER* bound to dimers, trimers and tetramers of the Kubox1 sequence (data not shown). Interestingly, binding of *DAYSLEEPER* to Kubox1 multimers and the *Ku70* upstream region fragment was dependent upon the presence of zinc ions (Fig. 1a). This is reminiscent of the

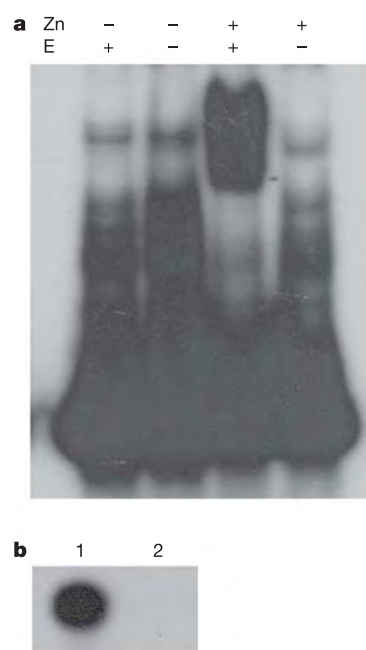


Figure 1 | *DAYSLEEPER* in vitro binding and expression in the mutant plant line. **a**, Gel retardation assay. Total protein extract (E) from *Escherichia coli* either containing *DAYSLEEPER* (+) or lacking the protein (-) was incubated with a radioactively labelled tetramer of Kubox1 in the presence (+) or absence (-) of additional Zn^{2+} ions. **b**, *DAYSLEEPER* expression in the mutant line. RT-PCR using 1 µg of total RNA was performed using *DAYSLEEPER* specific primers. The products were then diluted, blotted, and hybridized with a *DAYSLEEPER* probe. Lane 1, wild-type (ecotype Colombia) callus material; lane 2, callus derived from *DAYSLEEPER*^{-/-} plants.

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zinc dependent DNA binding of the Tag1 *hAT* transposase from *Arabidopsis*⁷. DAYSLEEPER is predicted to contain a C₂H₂ type BED-zinc finger⁸ between residues 70–118, which explains the zinc dependent binding. This was further demonstrated by site specific mutation of the following amino acids in the BED-type zinc finger (V70, H73, C88, C91, L109, H112 and C118) which led to loss of DNA binding capacity (see Supplementary Fig. S4). A BLAST search of the *Arabidopsis* genome was done to identify other genes with upstream sequences that have a consecutive stretch of at least 11 bp perfect homology with the 25 bp Kubox1 motif (see Supplementary Table S1). We isolated the promoter of one of these genes, At3g21215 (a putative RNA splicing protein similar to mec-8 from *D. melanogaster*⁹, see Supplementary Fig. S2), and used the one-hybrid assay to demonstrate that DAYSLEEPER also bound this promoter fragment. Thus, DAYSLEEPER binding is not restricted to the *Ku70* upstream regions alone and thus may be involved in the regulation of multiple genes.

In order to establish the importance of DAYSLEEPER for the plant, an *Arabidopsis* line (ecotype Colombia) was isolated in which the gene was disrupted by a T-DNA insertion located 24 bp downstream of the start codon (see Supplementary Fig. S5). In fact, several T-DNA copies inserted in a complex repeat structure were present, integrated at the locus. The DAYSLEEPER mutant was backcrossed twice to the wild type to segregate out unlinked T-DNA insertions. A plant (F₃ generation) resulting from this cross and containing only the T-DNA copies at the DAYSLEEPER locus was selected for further analysis. Seedlings homozygous (–/–) for the T-DNA insertion completely lacked DAYSLEEPER expression (Fig. 1b; lane 2) and showed a severe recessive phenotype (Fig. 2a, c). Such seedlings grew very slowly, and showed no expansion of the cotyledons or development of normal leaves or floral organs. The root remained short and had an excess of abnormal root hairs. The main and lateral root meristems appeared to have a normal organization when young, but as they matured became increasingly disorganized. After prolonged growth on plates, a few seedlings (1%) produced some photosynthetic tissue (Fig. 2b). Plants hemizygous (+/–) for the T-DNA in DAYSLEEPER appeared normal. We were able to complement the recessive phenotypes by introducing DAYSLEEPER under control of the constitutive cauliflower mosaic virus (CaMV) 35S promoter into +/– plants. Normal homozygous mutant offspring only developed if the complementation construct was present. This confirmed that the observed lack of seedling development was the result of the T-DNA insertion (data not shown).

During our complementation experiments we observed a dominant phenotype in lines with the highest DAYSLEEPER expression. To examine possible overexpression phenotypes further, we transformed wild-type (ecotype Colombia) plants with the CaMV 35S construct. Primary transformants (the T₁ generation) that showed the highest expression of DAYSLEEPER also showed developmental defects. Compared to plants transformed with the empty binary vector, these lines grew slowly, had delayed flowering, altered cauline leaves, fasciation and partial or total sterility (Fig. 2d). The flower phenotypes were also pronounced (Fig. 2e). The positioning and number of floral organs was correct, but sepal and petal growth was reduced, with the margins of the sepals often appearing petaloid. The overexpression phenotypes were even more severe in the successive generation. T₂ plants remained small and died at the seedling stage (data not shown).

Plants lacking DAYSLEEPER show severe growth defects, but is this accompanied by changes in expression of its putative target genes? Gene expression analysis on DAYSLEEPER homozygous seedlings was not attempted because such seedlings lack most plant organs, which complicates the analysis. In order to identify genes whose expression is indeed affected by DAYSLEEPER we took the following approach. DAYSLEEPER was overexpressed from an inducible promoter. The phenotypes of such plants after prolonged growth (14 days) on medium containing the inducer were identical to that

previously observed in the 35S::DAYSLEEPER plants (data not shown). However, to avoid changes in gene expression that are the result of the developmental abnormalities, normal one-week-old seedlings were induced in liquid medium for 24 h, after which RNA was isolated and then used in microarray experiments to detect changes in global gene expression. Such experiments showed that DAYSLEEPER overexpression resulted in the strong upregulation, in some cases more than 40-fold, of several genes (see Supplementary Table S2). In contrast, its effect on gene downregulation was more modest. We were unable to identify a perfect Kubox1 motif in any of the genes showing altered expression. These may either not be primary target genes or have DAYSLEEPER binding sites that are different from the Kubox1 motif.

In this experiment we also did not see changes in the expression of genes that did contain the Kubox1 motif in their upstream regions, and this included *Ku70*. In the plant the low level of expression from the endogenous DAYSLEEPER alleles may not be limiting for the expression of these genes, but a mutation in DAYSLEEPER may still lead to reduced expression. In fact, we have data from experiments using a *Ku70* promoter– β -glucuronidase (GUS) fusion that suggests that this may indeed be the case (data not shown). Further studies will be necessary to investigate whether DAYSLEEPER also plays a role in the direct regulation of these genes. On the basis of homology, DAYSLEEPER does not contain any extra domains with demonstrated regulatory activity. Therefore, DAYSLEEPER may interact

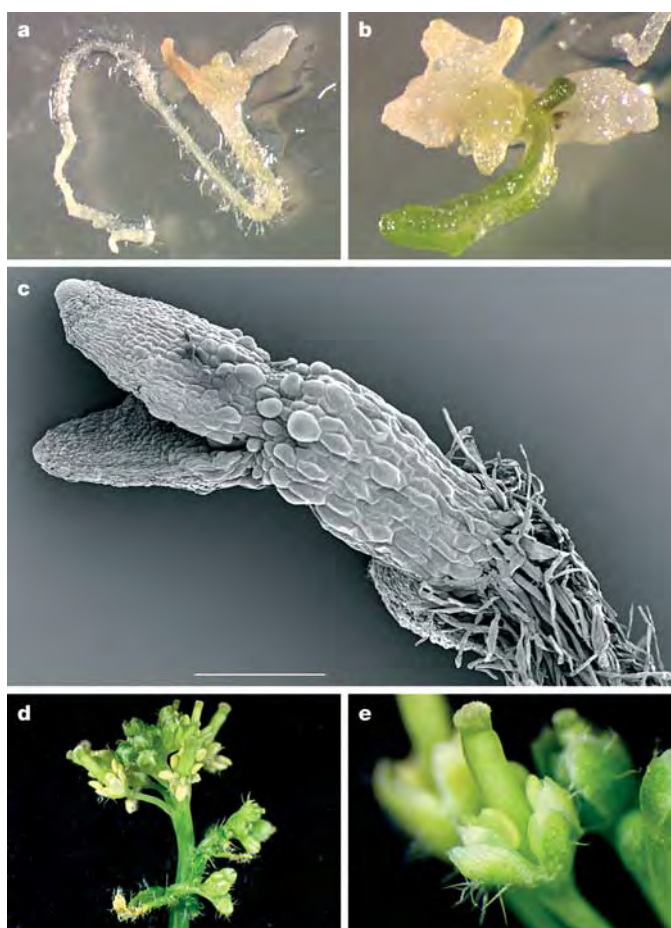


Figure 2 | Phenotypes of DAYSLEEPER knockout and overexpression plants. **a**, 4-week-old DAYSLEEPER^{–/–} seedlings. **b**, A small percentage of these seedlings do finally produce some photosynthetic tissue. **c**, Scanning electron micrograph of a 2-week-old DAYSLEEPER^{–/–} seedling (scale bar, 200 μ m). **d**, Inflorescence of T₁ plants overexpressing DAYSLEEPER, and **e**, the effect of overexpression on flower morphology.

with other proteins in order to facilitate changes in global gene expression. Interestingly, in a preliminary screen for DAYSLEEPER interactors, we identified a helicase and genes involved in chromatin remodelling (data not shown). Thus we propose that after DNA binding DAYSLEEPER alters gene expression by the recruitment of other cellular factors.

Evidence is accumulating that transposition can produce changes in gene regulation by providing *cis* acting regulatory sequences¹⁰ or by epigenetic silencing of nearby genes¹¹. Moreover, several retro-elements, such as *HET-A* and *TART*, provide a vital function for their host by lengthening shortened chromosome ends in *D. melanogaster*, a function normally performed by telomerase¹². It has also been shown that the retrotransposon L1 can have transcriptional effects on gene expression after insertion into intron sequences¹³. However, all these examples are the result of the transposition of an active element. *DAYSLEEPER* is unique in that it is the transposase-like sequence itself that is of benefit to the plant and is involved in gene regulation. Regulation of plant genes by transposase-like genes may be a more widespread phenomenon. Two known *Arabidopsis* mutations (*far1* and *fhy3*) that affect the plant response to far-red light map to genes sharing high homology with *Mu*DRA, the transposase of *Mutator* transposable elements¹⁴. Global transcription is also altered in these mutants^{14,15}. However, direct binding of *Far1* or *Fhy3* to a specific motif has not been demonstrated, and under normal light conditions these mutants do not show a phenotype. *DAYSLEEPER* is the first example of a gene derived from a transposon that plays an essential function in the host. Our work suggests that transposase-like sequences are not always just genetic fossils, as they apparently have the potential to evolve functions that are essential for plant growth and development.

METHODS

A 988-bp fragment of the *Ku70* upstream region (chromosome I: positions 5806790–5805801) was amplified and inserted as a *NotI/XbaI* fragment into the vector pINT1-HIS3NB (AY061966). One-hybrid experiments were performed as described¹⁶. Details of the mapping of the T-DNA insertion points and the gel retardation assays are included in the Supplementary Information. Plants overexpressing *DAYSLEEPER* were obtained by floral dip transformation of *Colombia* plants using *Agrobacterium* containing a CaMV 35S::At3g42170 construct. Transformed plants were selected on medium containing kanamycin (50 µg ml⁻¹) and timentin (100 µg ml⁻¹). Plants containing the inducible construct (pINDEX3::At3g42170) were selected on medium containing hygromycin (20 µg ml⁻¹) and timentin.

Received 7 September 2004; accepted 18 April 2005.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements We thank V. Lanquar, Z. Li, A. den Dulk-Ras and M. Corredor for technical assistance, and S. de Pater, P. Ouwerkerk and B. van der Zaal for comments on the manuscript. P.B. and P.H. are supported by the EU FP5 project PLANTREC, the Technology Foundation STW, the Applied Science Division of NWO, and the Technology Programme of the Ministry of Economic Affairs.

Author Information The sequence of the cDNA clone of At3g42170 has been submitted to GenBank (AY728267). Reprints and permissions information is available at npg.nature.com/reprintsandpermissions. The authors declare no competing financial interests. Correspondence and requests for materials should be addressed to P.H. (hooykaas@rubiim.leidenuniv.nl).

A substrate-specific inhibitor of protein translocation into the endoplasmic reticulum

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The segregation of secretory and membrane proteins to the mammalian endoplasmic reticulum is mediated by remarkably diverse signal sequences that have little or no homology with each other^{1,2}. Despite such sequence diversity, these signals are all recognized and interpreted by a highly conserved protein-conducting channel composed of the Sec61 complex^{3,4}. Signal recognition by Sec61 is essential for productive insertion of the nascent polypeptide into the translocation site⁵, channel gating⁶ and initiation of transport. Although subtle differences in these steps can be detected between different substrates^{7,8}, it is not known whether they can be exploited to modulate protein translocation selectively. Here we describe cotransin, a small molecule that inhibits protein translocation into the endoplasmic reticulum. Cotransin acts in a signal-sequence-discriminatory manner to prevent the stable insertion of select nascent chains into the Sec61 translocation channel. Thus, the range of substrates accommodated by the channel can be specifically and reversibly modulated by a cell-permeable small molecule that alters the interaction between signal sequences and the Sec61 complex.

A screen for inhibitors of the expression of cell adhesion molecules led to the identification of HUN-7293, a cyclodepsipeptide natural product that potently inhibits vascular cell adhesion molecule 1 (VCAM1) expression in endothelial cells⁹. The mechanism of action and molecular target of HUN-7293 are unknown. However, key structural features of HUN-7293 important for its activity have been defined by Boger and colleagues in a systematic analysis of 40 structural variants¹⁰. On the basis of this seminal study, we designed and synthesized a simplified analogue of HUN-7293, which we named 'cotransin' (Fig. 1a). We also synthesized 'nor-cotransin', a putatively inactive variant lacking a critical *N*-methyl moiety¹⁰.

To evaluate the activity of cotransin and nor-cotransin, primary human cells were stimulated under several conditions and characterized for the expression of 26 cell surface and secreted proteins¹¹. Cotransin, but not nor-cotransin, inhibited the stimulated expression of only two proteins, VCAM1 and P-selectin (Fig. 1b, c, and Supplementary Fig. S1). Analysis of either total secreted proteins (Fig. 1d) or cellular glycoproteins (Supplementary Fig. S2) from pulse-labelled COS-7 cells revealed a similarly selective effect. Cotransin therefore selectively inhibits the expression of a small subset of secretory and membrane proteins, including VCAM1.

Selective, potent and reversible inhibition of VCAM1 (Fig. 2a, b), but not other proteins (for example green fluorescent protein; data not shown) expressed heterologously from the constitutive cytomegalovirus promoter, indicated that the site of cotransin action is likely to be post-transcriptional and, possibly, post-translational. Indeed, proteasome inhibition during treatment with cotransin stabilized a non-glycosylated form of VCAM1 (Fig. 2a). This indicated that cotransin might cause newly synthesized VCAM1 to be degraded

rather than productively enter the secretory pathway, a conclusion consistent with data reported in a recent patent application¹².

To identify the step at which VCAM1 is rerouted from a biosynthetic to a degradative fate, its translation and translocation were analysed *in vitro* by using reticulocyte lysate containing rough endoplasmic reticulum (ER) microsomes (RM). Whereas the translation of VCAM1 was unaffected by cotransin, its translocation into RM was markedly inhibited (Fig. 2c). This is evident by a decrease in VCAM1 glycosylation and complete digestion of non-glycosylated product by exogenous protease (Fig. 2c, lanes 6 and 7). By contrast, translocation reactions containing nor-cotransin (or dimethylsulphoxide; DMSO) resulted in efficient glycosylation and protease protection of VCAM1 (Fig. 2c, lanes 3, 4, 8 and 9). Both VCAM1 translocation and its inhibition by cotransin were observed even when glycosylation was prevented (Fig. 2d), whereas the addition of cotransin after translocation had no effect on either glycosylation or protease protection (data not shown). In striking contrast to VCAM1, the translocation of pre-prolactin (pPrI) was unaffected by cotransin (Fig. 2c, d). Moreover, an analysis of model type I, type II and multi-spanning membrane proteins with cotransin showed no effect on their proper translocation, glycosylation, membrane insertion or topology *in vitro* (Supplementary Fig. S3). These data suggest that cotransin inhibits VCAM1 expression in cells by selectively preventing its cotranslational translocation into the ER.

The amino-terminal signal sequences that mediate the translocation of VCAM1 and pPrI into the ER are markedly different from one another (Fig. 2e). To test whether this difference underlies cotransin's substrate selectivity, we prepared chimaeric constructs in which the signals of VCAM1 and pPrI were fused to an otherwise non-translocated reporter. Although both signals directed efficient translocation of the reporter, only the VCAM1 fusion construct was inhibited by cotransin (Fig. 2e). Identical results were obtained when another reporter (the prion protein) was fused to the VCAM1 and pPrI signals (data not shown). Sensitivity to cotransin is therefore determined primarily by the signal sequence. Although these results indicate that the VCAM1 signal sequence could be the direct target of cotransin, examination of additional cotransin-sensitive and cotransin-resistant substrates indicated that this is improbable (Supplementary Table 1). Comparison of their signal sequences failed to reveal either a consensus cotransin-binding motif shared by the sensitive substrates or the basis for cotransin resistance. We therefore focused on the targeting and translocation machinery, components of which are known to interact with diverse signal sequences, as potential targets of cotransin.

To identify the step of protein translocation inhibited by cotransin, we analysed interactions between VCAM1 nascent chains and the translocation machinery by chemical crosslinking. In the cytosol, 145-residue ribosome-nascent chain complexes (RNCs) of VCAM1 formed crosslinks with several proteins, none of which were affected

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by cotransin (Fig. 3a, lanes 7–9). This pattern of crosslinks changed when the RNCs were prepared in the presence of RM, indicating a clear shift in the environment surrounding the nascent chain (Fig. 3a, lanes 10–12). As expected, a major cytosolic crosslink was identified by immunoprecipitation as SRP54 (Fig. 3a, lanes 7–9), the signal sequence-binding subunit of SRP^{13,14}. This interaction was unperturbed by cotransin (Fig. 3a, lane 8) but was lost after the addition of microsomes (lanes 10–12). Taken together, the identical crosslinking patterns before targeting, equal efficiencies of SRP54 interaction and equal efficiencies of SRP release are evidence that cotransin has no effect on the steps preceding the SRP receptor (SR)-mediated transfer of RNCs to the translocation channel. These results also argue against the VCAM1 signal sequence itself as the direct target of cotransin.

Instead, the first cotransin-sensitive step occurs at the membrane.

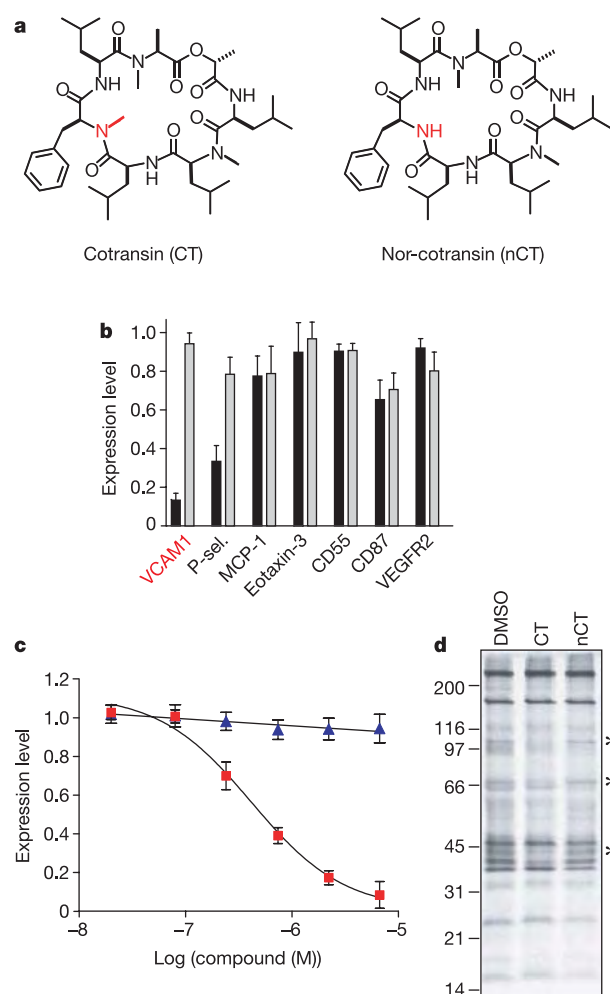


Figure 1 | Cotransin inhibits expression of a subset of secreted and membrane proteins. a, Structures of cotransin and nor-cotransin.

b, Primary human endothelial cells were treated with DMSO, 2 μ M cotransin (black bars) or 2 μ M nor-cotransin (grey bars) and stimulated with 5 ng ml⁻¹ interleukin-4 and 10 μ M histamine. After 24 h, cell surface proteins were quantified by enzyme-linked immunosorbent assay. Protein expression levels were normalized to DMSO controls (means $\pm$ s.e.m.; $n = 6$). Additional results are shown in Supplementary Fig. S1. MCP-1, monocyte chemoattractant protein-1; P-sel., P-selectin; VEGFR2, vascular endothelial growth factor receptor-2. **c**, Dose-response curve showing the effect of cotransin (red squares) and nor-cotransin (blue triangles) on VCAM1 expression (means $\pm$ s.e.m.; $n = 6$). The IC₅₀ is about 0.5 μ M, compared with about 1 nM for HUN-7293 (refs 9, 10). **d**, Effect of cotransin (5 μ M) on total secreted proteins from pulse-labelled COS-7 cells. Asterisks indicate secreted proteins whose levels are reduced by cotransin. Molecular masses are in kDa.

Signal sequence cleavage, an indicator of nascent chain access to signal peptidase on the luminal side of the membrane, was not observed with cotransin (Fig. 3a, lanes 5 and 11). Furthermore, cotransin, but not nor-cotransin, significantly altered the pattern of crosslinks between RNCs and ER proteins (Fig. 3a, lanes 10–12; Fig. 3b, lanes 4–6). Differences in crosslinking were apparent with RNCs of several different lengths and with both lysine-reactive and cysteine-reactive crosslinkers (Fig. 3a, b, and data not shown). Immunoprecipitation studies identified the primary differences (Fig. 3b). RNCs in DMSO and nor-cotransin samples crosslinked efficiently to Sec61 α and luminal chaperones (such as protein disulphide isomerase; PDI), but not to Sec61 β . By contrast, RNCs synthesized in the presence of cotransin showed diminished Sec61 α and PDI crosslinks but enhanced Sec61 β crosslinks. RNCs are therefore in close proximity to the Sec61 complex with or without cotransin; however, their orientation with respect to Sec61 subunits is significantly perturbed by cotransin.

Sedimentation, protease protection and transport assays of translocation intermediates led to similar conclusions. In these experiments, RNCs in the cotransin, nor-cotransin and DMSO

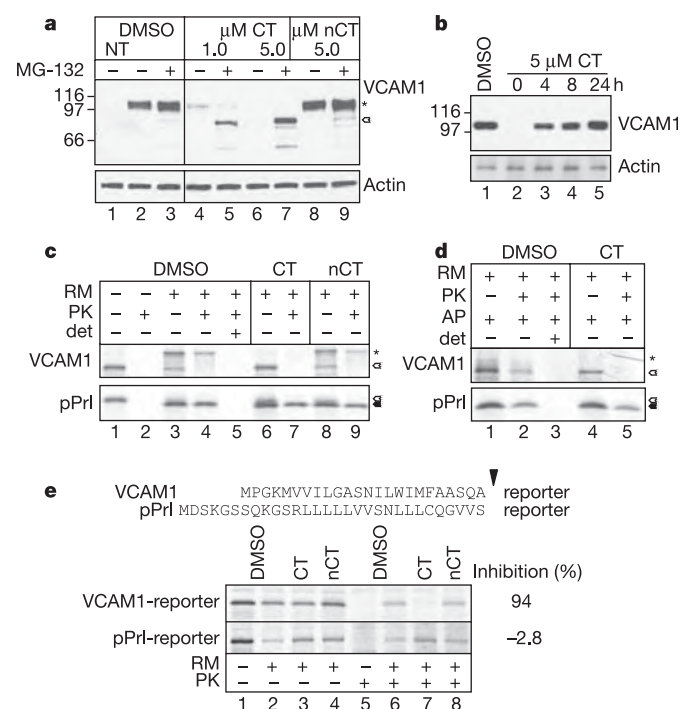


Figure 2 | Cotransin inhibits cotranslational translocation. a, COS-7 cells were transfected with a VCAM1 expression plasmid or not transfected (NT) and treated with cotransin (CT) or nor-cotransin (nCT). The proteasome inhibitor MG-132 (5 μ M) was included where indicated. After 24 h, cell lysates were analysed by immunoblotting with anti-VCAM1 or anti-actin antibodies. **b**, Cells were transfected as in **a**, except that cotransin was removed from the medium after 24 h. Cells were harvested at specified time points and analysed for VCAM1 expression. Molecular masses in **a** and **b** are in kDa. **c**, Transcripts encoding VCAM1 or pre-prolactin (pPrI) were translated in the presence of [³⁵S]methionine and, where indicated, rough ER microsomes (RM). Translation reactions also contained 10 μ M cotransin, 10 μ M nor-cotransin or DMSO. Equal aliquots of the translated material were left untreated or treated with PK in the presence or absence of Triton X-100 (det). Samples were separated by SDS-PAGE and analysed by autoradiography. The positions of precursor (open arrow), signal-cleaved (solid arrow) and glycosylated (asterisk) species are indicated. **d**, As in **c**, except that a peptide inhibitor of glycosylation (AP) was included in the translation reactions. **e**, VCAM1 and pPrI signal sequences (shown) were appended to the N terminus of a Gal4-NF κ B fusion protein ('reporter') and the resulting constructs were tested in cell-free translocation assays as in **c** (quantified by phosphorimager analysis).

samples quantitatively achieved salt-resistant binding to microsome, a previously defined indicator of tight interaction between ribosome and translocon after transfer from SRP^{5,15,16}. Treatment of these salt-resistant translocation intermediates with protease showed that cotransin-treated RNCs were accessible to exogenous protease (Fig. 3c, lane 8), whereas the control samples were protease-protected in the absence, but not the presence, of detergent (lanes 3, 4 and 12). The cotransin-treated RNCs remained accessible to protease even after release from the ribosome with puromycin (Fig. 3c, lane 10), indicating that although they were at the translocon, they could not translocate into the lumen. Protease accessibility and inhibition of translocation were observed only if cotransin was present during the assembly of translocation intermediates. Addition after translocation intermediate assembly had no effect (data not shown), indicating that once nascent chains have initiated translocation, they are no longer influenced by cotransin. Cotransin therefore acts at a step after the targeting and transfer of RNCs to the translocon, but before the nascent chains have access to the ER lumen.

The post-targeting step inhibited by cotransin involves insertion of the nascent chain into the translocation channel and opening of the channel towards the lumen, and proteins engaged in this step are potential molecular targets of cotransin. These include the Sec61 complex⁵ and several accessory components of the translocon (such as TRAM^{15,17}, the TRAP complex¹⁸, the Sec62/63 complex^{19,20} or BiP^{20–23}). However, removal of these and other components from the membrane or lumen had little effect on VCAM1 translocation beyond that observed for pPrl (Supplementary Figs S4 and S5). These accessory components are therefore largely dispensable for

VCAM1 translocation and are unlikely to be targets for cotransin inhibition. The only protein whose depletion prevented VCAM1 translocation was the Sec61 complex (Supplementary Fig. S5), a component also essential for pPrl translocation²⁴.

Because every known accessory protein previously implicated in translocation was represented in these depletions, the target of cotransin is likely to be a novel protein or the Sec61 complex itself. To explore the latter possibility, we prepared proteoliposomes containing only the Sec61 complex and SR (Supplementary Fig. S6), the minimal machinery required for protein translocation²⁴. VCAM1 and pPrl were both translocated into the lumen of these minimal proteoliposomes (at reduced efficiency); however, only VCAM1 was inhibited by cotransin (Fig. 4a, b). Given that targeting to the translocon is unaffected by cotransin (Fig. 3a), these results suggest that cotransin acts to prevent signal sequence-dependent gating of the Sec61 translocation channel.

To test this hypothesis directly, we examined the interaction between VCAM1 or pPrl nascent chains and purified Sec61. A

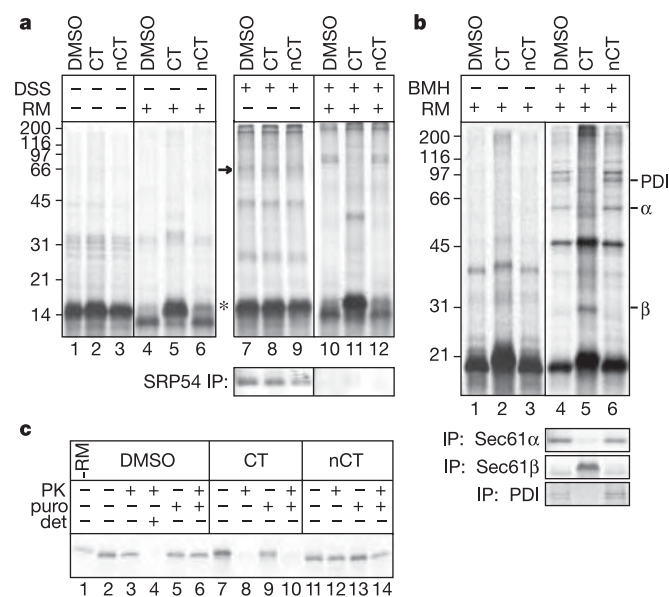


Figure 3 | Cotransin inhibits insertion of VCAM1 into the translocation channel. **a**, VCAM1 nascent chains of 145 residues (145-mers) were synthesized in the absence or presence of RM, isolated by centrifugation and subjected to crosslinking with disuccinimidyl suberate (DSS). Samples were separated by SDS-PAGE and analysed by autoradiography. Crosslinks to SRP54 (arrow, lanes 7–9) were confirmed by immunoprecipitation (IP). The position of non-signal-cleaved VCAM1 is indicated with an asterisk. **b**, VCAM1 180-mers were translated in the presence of RM, isolated by centrifugation through a high-salt cushion, and subjected to crosslinking with bismaleimido-hexane (BMH). Crosslinks to Sec61 α , Sec61 β and PDI (marked by arrows) were confirmed by immunoprecipitation. Molecular masses in **a** and **b** are in kDa. **c**, VCAM1 180-mers were translated in the presence of RM and isolated as in **b**. Equal aliquots were treated with puromycin (puro) and PK as indicated. Cotransin or non-cotransin was included during the translation reaction at a final concentration of 25 μ M. Det, detergent (Triton X-100).

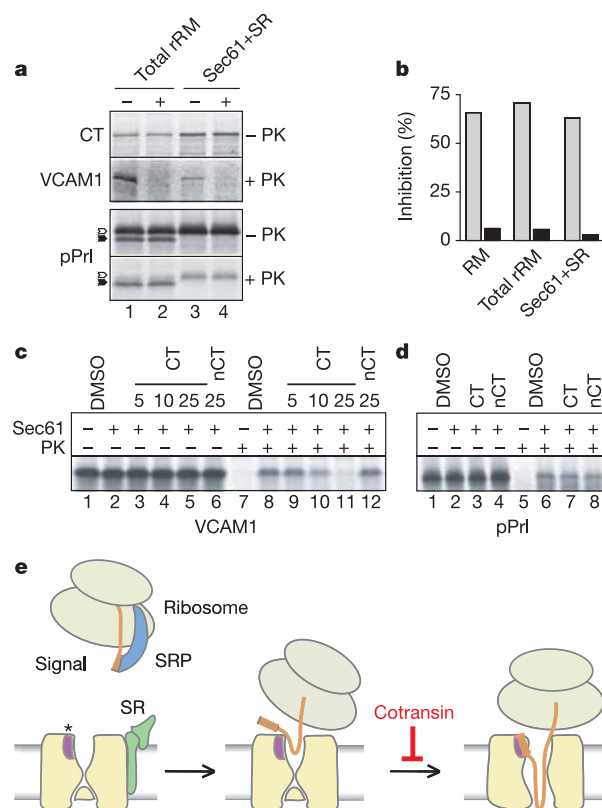


Figure 4 | Cotransin prevents signal sequence recognition by the Sec61 complex. **a**, Full-length VCAM1 or pPrl was translated in the presence of proteoliposomes containing total ER membrane proteins (total rRM) or purified Sec61 complex and SRP receptor (Sec61/SR). Where indicated, cotransin (10 μ M) was included. **b**, Translocation data from panel **a** were quantified by phosphorimager analysis. Grey bars, VCAM1; black bars, pPrl. **c**, VCAM1 145-mer RNCs were isolated by centrifugation through a high-salt cushion. RNCs were incubated with purified Sec61 complex in the presence of compounds as indicated. Samples were left untreated or treated with PK. Concentrations of cotransin and non-cotransin are shown in μ M. **d**, As in **c**, except that pPrl 145-mers were used. Cotransin and non-cotransin were used at a final concentration of 25 μ M. **e**, Model of cotransin activity. SRP-mediated targeting of the RNC to a Sec61-containing translocon in the ER membrane is unaffected by cotransin. Rather, cotransin blocks signal sequence-dependent insertion of the nascent chain into the translocation channel and simultaneous opening of the pore towards the lumen of the endoplasmic reticulum.

productive interaction between RNCs and Sec61 shields the nascent chain from protease digestion because of its insertion into the translocation channel^{5,25}. Using this assay, we found that purified 145-mer RNCs of either VCAM1 or pPrl, which are readily digested by protease, become protected on addition of the Sec61 complex (Fig. 4c, compare lanes 7 and 8; Fig. 4d, compare lanes 5 and 6). Remarkably, insertion of the VCAM1 nascent chain into the Sec61 channel is selectively inhibited by cotransin (Fig. 4c, lanes 11 and 12). Cotransin had no effect on the pPrl nascent chain (Fig. 4d). Thus, cotransin acts in a signal sequence-discriminatory manner to prevent the nascent chain from interacting productively with the Sec61 channel (Fig. 4e).

In this study we have characterized cotransin, the first small-molecule modulator of protein translocation into the ER. Cotransin inhibits translocation in a signal sequence-dependent manner, despite targeting a translocation channel utilized by all secretory and membrane proteins. Identification of the exact features in the signal that confer sensitivity (and resistance) to cotransin must await systematic mutagenesis studies. However, it is already apparent that differences between natural signal sequences can markedly influence sensitivity to cotransin (Supplementary Table 1). This implies that signal sequence variation can be exploited to modulate the functional expression of secreted and membrane proteins at their point of entry into the secretory pathway.

The precise molecular mechanism by which cotransin inhibits nascent chain insertion and gating of the Sec61 channel for select substrates is not known. One possibility is that cotransin stabilizes the channel in a closed conformation such that gating by low-affinity signal sequences is inefficient. Alternatively, cotransin might directly or allosterically alter the topography of a signal sequence binding site, postulated to involve transmembrane helices 2 and 7 of Sec61 α ^{26,27}. In this model, the altered binding site would be less accommodating or flexible, resulting in increased substrate selectivity. Deciphering the molecular details of how cotransin influences the signal sequence interaction with the Sec61 channel is likely to shed light on the mechanism by which unrelated signal sequences gate a communal translocation channel. Cotransin thus provides a powerful new probe for investigating the molecular function of a protein-conducting channel as well as a selective, reversible method of inhibiting cotranslational translocation in living cells.

METHODS

Constructs. The coding region for human VCAM1 was amplified by polymerase chain reaction with reverse transcription (RT-PCR) from total RNA isolated from tumour necrosis factor- α -stimulated A549 cells (gift from H. Luecke) and cloned into the pcDNA3.1 vector (Invitrogen). Bovine pPrl in the SP64 vector (Promega) has been described previously¹⁸. The Gal4-NFkB coding region (from plasmid pBD-NFkB (Stratagene)) containing a carboxy-terminal KDEL sequence was amplified by PCR and subcloned into signal sequence cassettes⁸ containing the VCAM1 or pPrl signal sequence to generate the signal-Gal4-NFkB constructs. All constructs were verified by sequencing.

Antibodies and proteins. Antibodies against Sec61 β were described previously¹⁸. Antibodies against Sec61 α were a gift from R. Gilmore. Antibodies against VCAM1, PDI, actin and SRP54 were purchased from Santa Cruz, Stressgen, Sigma and BD Biosciences, respectively. Mammalian Sec61 complex and SR were purified from canine RM as described previously²⁴. Immunoblotting relative to a titration of starting RM at a defined concentration of 1 equivalent (equiv) μl^{-1} was used to determine the concentration of purified proteins. Abundances of about 2 pmol equiv⁻¹ and about 0.2 pmol equiv⁻¹ for the Sec61 complex and SR, respectively, have been established in previous studies²⁴.

Small molecule synthesis. Cotransin and nor-cotransin were synthesized by adapting the published synthesis of HUN-7293 (ref. 28) and were characterized by ¹H-NMR and mass spectrometry.

Mammalian cell culture and transient expression of VCAM1. A detailed description of cell-based models of inflammation is given in Supplementary Methods. COS-7 cells were maintained in accordance with standard procedures. Pulse-labelling of secreted proteins was performed on cells preincubated for 60 min at 37 °C with cotransin or nor-cotransin (5 μM) in medium lacking

methionine, cysteine and serum. After being labelled with 400 $\mu\text{Ci ml}^{-1}$ [³⁵S]methionine/cysteine for 30 min, proteins in the medium were collected by precipitation with 15% trichloroacetic acid, washed in acetone, dissolved in 1% SDS, 0.1 M Tris-HCl pH 8, and analysed by SDS-polyacrylamide-gel electrophoresis (SDS-PAGE) and autoradiography. VCAM1 expression analysis was performed 24 h after transfection with Lipofectamine 2000 (Invitrogen). Where indicated, cotransin, nor-cotransin or MG-132 (5 μM ; Calbiochem) was added 5 h after transfection. Cells were harvested in 1% SDS, 0.1 M Tris-HCl pH 8 and analysed by SDS-PAGE and immunoblotting.

Cell-free translocation assays. *In vitro* transcription, translation, translocation, and protease protection assays were performed as described previously^{8,15}. Truncated transcripts lacking a stop codon were synthesized from PCR-generated templates⁵. Translations were at 32 °C for 60 min (full-length constructs) or for 20 min (truncated constructs). Nascent chains were isolated at 4 °C by sedimentation in a TLA 100.3 rotor (75,000 r.p.m. for 45 min (for RNCs) or 70,000 r.p.m. for 10 min (for membrane-bound RNCs)) through a sucrose cushion (100 μl , 0.5 M) in PSB buffer (50 mM HEPES pH 7.4, 150 mM potassium acetate, 5 mM magnesium acetate). Translation reactions to measure salt-resistant binding were adjusted to 0.5 M potassium acetate and sedimented through a 0.5 M sucrose cushion containing 0.5 M potassium acetate in PSB. Isolated nascent chains were resuspended in PSB containing 0.25 M sucrose before further manipulations. Treatment with puromycin (1 mM; Calbiochem) was for 15 min at 25 °C (Fig. 3c). In Fig. 4c, d, nascent chains were isolated in the absence of compound, then resuspended in the presence of cotransin or nor-cotransin (25 μM) before treatment with proteinase K (PK). Inhibition of glycosylation was with a competitive peptide inhibitor (NH₂-Asp-Tyr-Thr-COOH; California Peptide Research) at 100 μM (Fig. 2d). Quantitative analysis of translocation experiments used a Typhoon 9400 phosphorimager (Amersham) with accompanying software.

Crosslinking assays. Crosslinking with 145-mers was with 0.5 mM disuccinimidyl suberate (Pierce) at 23 °C for 30 min; the reaction was quenched with 0.1 M Tris-HCl pH 8.0, 1% saponin (Sigma), 10 mM EDTA, 50 $\mu\text{g ml}^{-1}$ RNase A (Sigma), then added to 1% SDS, 0.1 M Tris-HCl pH 8 and heated to 37 °C. Crosslinking with 180-mers (isolated in the presence of high salt concentration) was with 50 μM bismaleimidoethane (Pierce) at 23 °C for 15 min; the reaction was quenched with 100 mM 2-mercaptoethanol, then added to 1% SDS, 0.1 M Tris-HCl pH 8 and heated to 37 °C. Immunoprecipitation with the indicated antibodies was performed after the dilution of samples tenfold with 1% Triton X-100, 50 mM HEPES pH 7.4, 100 mM sodium chloride.

Reconstituted proteoliposomes. Glycoprotein-depleted and Q Sepharose-depleted proteoliposomes were prepared as described previously¹⁸. Sec61-depleted proteoliposomes and proteoliposomes containing purified Sec61 and SR were prepared as described²⁴.

Assays with RNCs and purified Sec61 complex in detergent solution. Nascent 145-mer chains translated in the absence of compound and isolated in the presence of high salt concentration were resuspended in half the original volume of 50 mM HEPES pH 7.4, 25 mM potassium acetate, 2 mM magnesium acetate, 0.3% DeoxyBigCHAP (Calbiochem) and 25 μM cotransin or nor-cotransin where indicated. Purified Sec61 complex was added to a final concentration of about 500 nM and the samples were incubated at 32 °C for 20 min before transfer to ice for protease protection assays. Proteolysis reactions were with 0.5 mg ml⁻¹ PK for 60 min on ice as described¹⁸.

Received 18 February; accepted 18 May 2005.

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- Supplementary Information** is linked to the online version of the paper at www.nature.com/nature.
- Acknowledgements** E.J.K. thanks E. Rosler, S. Privat, D. Nguyen, J. Melrose, M. Fischer and M. Dea for technical assistance. R.S.H. thanks A. Sharma and M. Alken for technical assistance, and N. Rane and E. Snapp for discussions. J.L.G. thanks Z. Knight for discussions and critical reading of the manuscript. J.L.G. is supported by an NSF graduate fellowship. J.T. is a Searle Scholar and a fellow of the Alfred P. Sloan Foundation.
- Author Information** Reprints and permissions information is available at npg.nature.com/reprintsandpermissions. The authors declare no competing financial interests. Correspondence and requests for materials should be addressed to R.S.H. (hegder@mail.nih.gov) or J.T. (taunton@cmp.ucsf.edu).

LETTERS

Selective inhibition of cotranslational translocation of vascular cell adhesion molecule 1

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Increased expression of vascular cell adhesion molecule 1 (VCAM1) is associated with a variety of chronic inflammatory conditions, making its expression and function a target for therapeutic intervention^{1–3}. We have recently identified CAM741, a derivative of a fungus-derived cyclopeptolide that acts as a selective inhibitor of VCAM1 synthesis in endothelial cells. Here we show that the compound represses the biosynthesis of VCAM1 in cells by blocking the process of cotranslational translocation, which is dependent on the signal peptide of VCAM1. CAM741 does not inhibit targeting of the VCAM1 nascent chains to the translocon channel but prevents translocation to the luminal side of the endoplasmic reticulum (ER), through a process that involves the translocon component Sec61 β . Consequently, the VCAM1 precursor protein is synthesized towards the cytosolic compartment of the cells, where it is degraded. Our results indicate that the inhibition of cotranslational translocation with low-molecular-mass compounds, using specificity conferred by signal peptides, can modulate the biosynthesis of certain secreted and/or membrane proteins. In addition, they highlight cotranslational translocation at the ER membrane as a potential target for drug discovery.

During the search for inhibitors of VCAM1 expression, we identified the fungus-derived cyclic heptadepsipeptide (cyclopeptolide) CAM741, a decanopropyl ester of the natural compound HUN-7293 (Fig. 1a), which potently and selectively inhibits VCAM1 protein expression on human endothelial cells relative to intercellular adhesion molecule 1 (ICAM1) and E-selectin^{2,4,5}. Inhibition of VCAM1 expression occurred post-transcriptionally, because VCAM1 messenger RNA levels were not downregulated by the compound. Moreover, VCAM1 protein half-life was not affected, indicating an effect of CAM741 on protein translation or transport of VCAM1 to the cell surface (J.B. & S.W., unpublished observation).

To determine whether CAM741 has a direct effect on VCAM1 protein production, we transfected HEK-293 cells with VCAM1 or E-selectin expression plasmids and incubated the cells with the compound. VCAM1 and E-selectin were expressed as fully glycosylated proteins, as deduced from their apparent molecular masses (Fig. 1b) and digestion with endoglycosidase F (Fig. 2b). VCAM1 glycoprotein expression was dose-dependently inhibited by CAM741, whereas E-selectin expression was unaffected at the concentrations tested (Fig. 1b).

Endothelial cell adhesion molecules VCAM1, ICAM1 and E-selectin are type I transmembrane proteins expressed as precursor proteins containing an amino-terminal signal peptide (SP), which targets the nascent polypeptide to the ER membrane for cotranslational translocation^{6–8}. To test the effect of the drug on this element of

the VCAM1 biosynthesis pathway, we performed translations of VCAM1, ICAM1 and E-selectin full-length complementary DNAs *in vitro* in a cell-free reticulocyte lysate system in the absence or presence of canine pancreatic microsomal membranes. In the absence of membranes, single protein bands for VCAM1, ICAM1 and E-selectin were detected (Fig. 3b). Proteins that translocated efficiently to the lumen of the ER were glycosylated, as indicated by their higher molecular mass, resistance to degradation by added proteases and sedimentation with the membranes. The residual non-translocated, unglycosylated polypeptides were digested by added proteases and remained in the supernatant (Fig. 3c, d). In the presence of CAM741, appearance of the glycosylated form of VCAM1 was dose-dependently inhibited, whereas concentrations of the non-translocated form increased. In contrast, translocation of ICAM1 was inhibited at 20–30-fold higher concentrations, whereas

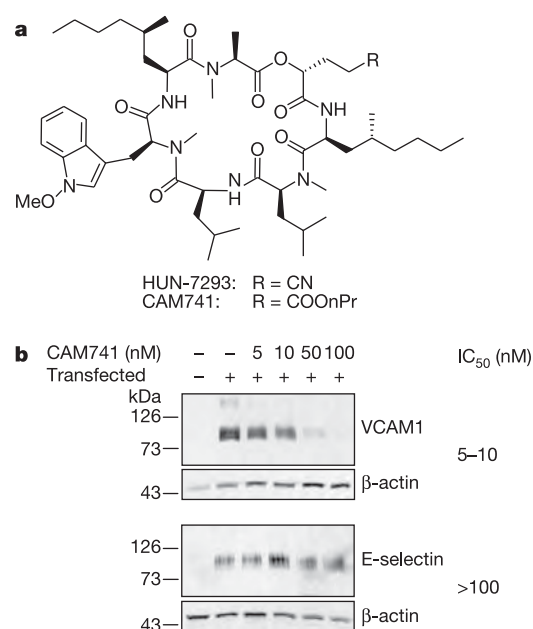


Figure 1 | CAM741 dose-dependently inhibits VCAM1 glycoprotein expression in transiently transfected cells. a, Structure of the cyclopeptolide CAM741. **b**, Western blot analysis of lysates from HEK-293 cells transiently transfected with VCAM1 or E-selectin expression plasmids and incubated with increasing concentrations of CAM741. IC₅₀ values, based on densitometric analysis, were calculated after normalization to β -actin.

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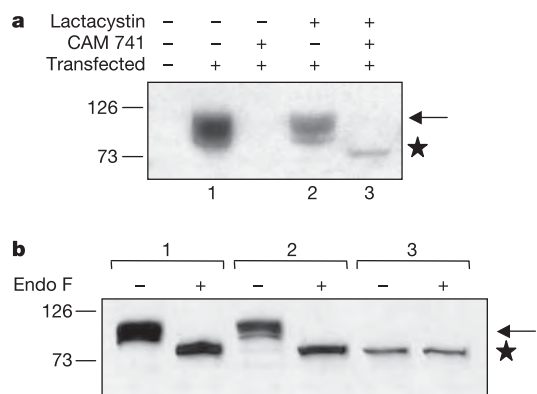


Figure 2 | Overexpressed VCAM1 is degraded by intracellular proteases in the presence of CAM741. **a**, Western blot analysis of lysates from HEK-293 cells transiently transfected with the VCAM1 expression plasmid either untreated or treated for 24 h with 100 nM CAM741 or 5 μ M lactacystin, or treated simultaneously with CAM741 and lactacystin. **b**, Western blot analysis of cell lysates 1, 2 and 3 of the transient transfection experiment shown in **a**, either untreated or deglycosylated with endoglycosidase F (Endo F). Arrow, glycosylated VCAM1 protein; star, non-glycosylated VCAM1 protein. Numbers at the left are molecular masses in kDa.

E-selectin translocation was not inhibited by the compound at any concentration tested (Fig. 3). These results indicate efficient translocation of all three nascent polypeptide chains across the microsomal membranes and show that CAM741 selectively inhibits the translocation of VCAM1. Although VCAM1 seems more sensitive to CAM741 in the cellular system, the relative selectivity of the compound *in vitro* matches that determined in transfected cells.

SPs have a decisive function in the initial targeting to, and translocation across, the ER membrane of proteins using the classical ER–Golgi transport pathway^{6–12}. In addition, for integral type I transmembrane proteins such as adhesion molecules, insertion into the ER membrane is mediated by their carboxy-terminal transmembrane (TM) domains. To establish which domain of VCAM1 is required for drug sensitivity, we generated VCAM1 N-terminal and C-terminal deletion mutants, as shown in Fig. 4a. The effect of CAM741 on these constructs was tested by translocation assays

in vitro and western blot analysis. The mutant VCAM1 molecule lacking the 17 N-terminal amino acids of the SP (Δ_{1-17} SPVCAM1) was, as expected, translocation deficient. No glycosylated form was generated in the cell-free system, and consequently CAM741 had no effect (Fig. 4b, left panel). This mutant form was also undetectable in transfected cells, probably because of mislocalization of the nascent polypeptide to the cytosol, resulting in degradation. VCAM1 lacking parts of the C terminus containing the TM (VCAM1(-TM)) domain was efficiently translocated in the cell-free system and synthesized in transiently transfected cells. In addition, it was as sensitive to the compound as full-length VCAM1 protein (Fig. 4b). These results show that the TM domain is not required for activity of CAM741 and indicate a function of the SP in drug sensitivity.

We then examined the effect of CAM741 on the expression of two chimaeric fusion constructs combining the SPs and mature sequences of VCAM1 and E-selectin (Fig. 4a). The VCAM1 mutant molecule containing the SP from E-selectin (E-sel/VCAM1) was resistant, whereas translocation of E-selectin fused to the VCAM1 SP (VCAM1/E-sel) was inhibited by CAM741, although this mutant was less sensitive than wild-type VCAM1. These effects were observed in both cell-free translocation and cellular glycoprotein synthesis assays (Fig. 4b). Further analysis of the VCAM1 SP showed that the presence of additional amino acids of the VCAM1 mature domain greatly enhanced the sensitivity to the compound, whereas parts of the N-terminal region of the VCAM1 SP were dispensable for both translocation and drug sensitivity (Supplementary Fig. 1). Transient transfections of HEK-293 cells with the VCAM1 SP fused to human placental secreted alkaline phosphatase (SEAP) demonstrated that the secretion of SEAP was dose-dependently inhibited by CAM741. In addition, we showed that the VCAM1 SP plus the first amino acid of the VCAM1 mature domain represents the minimal region for full sensitivity to CAM741 (Supplementary Table 1).

To determine the level at which CAM741 inhibits VCAM1 translocation, we used truncated mRNAs lacking a stop codon to create translocation intermediates. The use of VCAM1 nascent chains of 112 amino-acid residues resulted in comparable targeting and tight complex formation in the control and CAM741-treated reactions, and in both cases the nascent chains remained protected from protease. However, after release of the nascent chains from the ribosome by puromycin, translocation was observed only in the absence of compound, as witnessed by SP cleavage and protection

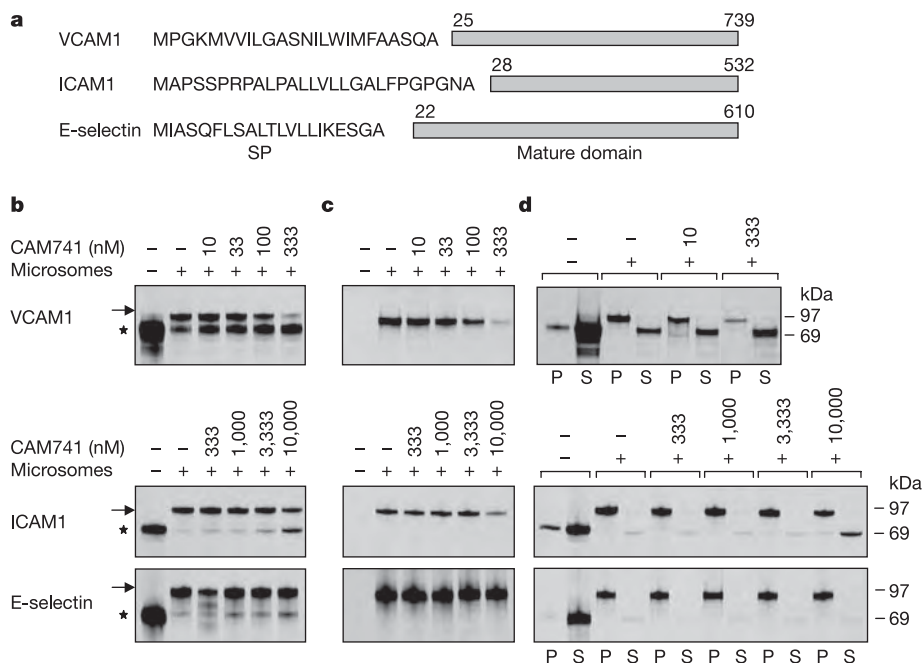


Figure 3 | CAM741 dose-dependently inhibits cotranslational translocation of VCAM1.

a, Schematic representation of the constructs used. **b–d**, Translocation of [³⁵S]methionine-labelled VCAM1, ICAM1 or E-selectin *in vitro* in the presence of increasing concentrations of CAM741. Translation/translocation products were analysed either directly (**b**), after treatment with proteinase K (**c**) or after sucrose cushion centrifugation (**d**). S, supernatant; P, microsomal pellet; arrow, glycosylated proteins; star, non-glycosylated proteins.

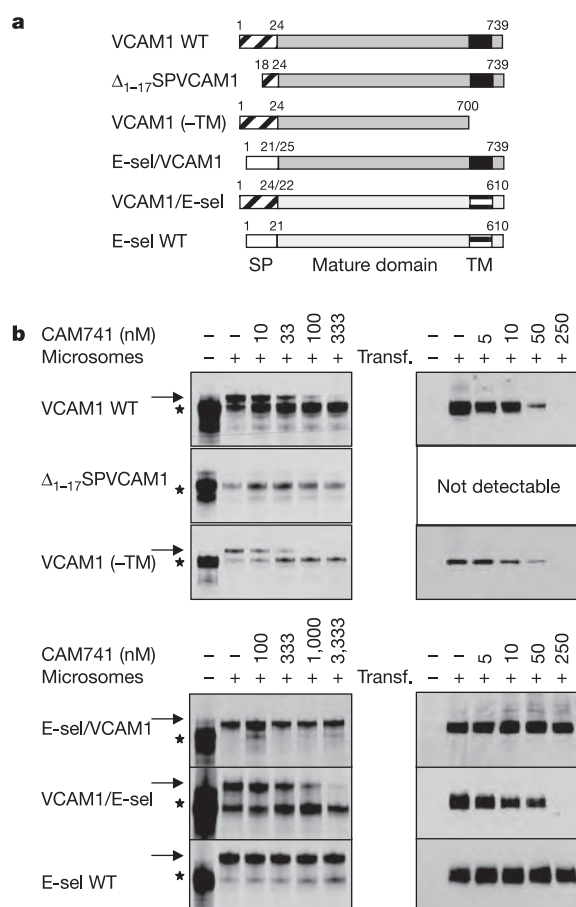


Figure 4 | VCAM1 SP is crucial for conferring cycloleptoid sensitivity.

a, Schematic representation of the deletion and chimaeric fusion constructs used. WT, wild type. **b**, Left: translocation of [35 S]methionine-labelled VCAM1, VCAM1 deletion constructs, E-selectin or chimaeric fusion constructs *in vitro* in the presence of increasing concentrations of CAM741. Arrow, glycosylated proteins; star, non-glycosylated proteins. Right: western blot analysis of lysates from COS-7 cells transiently transfected (transf.) with VCAM1, VCAM1 deletion constructs, E-selectin or chimaeric fusion constructs in the presence of increasing concentrations of CAM741. Δ_{1-17} SPVCAM1, VCAM1 mutant lacking the 17 N-terminal amino acids of the SP; VCAM1(-TM), VCAM1 mutant lacking parts of the C terminus containing the TM domain; E-sel/VCAM1, VCAM1 fused to the E-selectin SP; VCAM1/E-sel, E-selectin fused to the VCAM1 SP.

from protease (Fig. 5a). When we used VCAM1 nascent chains of 162 residues, SP cleavage was observed during targeting, whereas in the presence of CAM741, targeting was again obtained but there was no SP cleavage or protection from protease. After release of the nascent chains from the ribosome by puromycin, translocation was observed in the control reaction, whereas in the presence of CAM741 the targeted nascent chains were sedimented with the microsomal membranes but were not translocated (Fig. 5a). These data indicate that, although the targeting step was not inhibited by CAM741, translocation of the VCAM1 nascent chains towards the luminal side of the ER was prevented and the growing polypeptide chains were synthesized towards the cytosolic side of the ER. The notion that the VCAM1 nascent chains are indeed inserted into the translocon in the presence of CAM741 was further supported by the observation that after crosslinking with *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS) and immunoprecipitation, the nascent chains interacted with the translocon component Sec61 α (Fig. 5b). This observation confirms that CAM741 inhibits VCAM1 translocation at a post-targeting step.

To determine whether CAM741 could modulate early events in translocation¹³, we used short nascent chains of 81–112 residues. Crosslinking of targeted nascent chains with the homobifunctional thiol-reactive crosslinker bis-maleimido-hexane (BMH) revealed different crosslinked products between control and CAM741-treated reactions. One main product formed in the presence of compound was found by immunoprecipitation to contain the translocon component Sec61 β ¹⁴ (Fig. 5c). This close association reflected by the crosslink product could be either the cause or the result of incorrect insertion of the VCAM1 nascent chains into the translocon, preventing further translocation. Although the exact role of Sec61 β is not fully resolved, our results show involvement of this protein in the action of the compound. In addition, they indicate that CAM741 will serve as a useful tool for further dissection of the translocation process.

Last, we determined that CAM741 also diverts synthesis of VCAM1 towards the cytosolic compartment in living cells. HEK-293 cells were transiently transfected with the VCAM1 expression plasmid and treated with CAM741 and lactacystin, to block the ubiquitin proteasome pathway responsible for the degradation of misfolded proteins¹⁵. Whereas treatment with CAM741 alone efficiently inhibited the formation of glycosylated VCAM1, the addition of lactacystin to CAM741-treated cells resulted in the appearance of unglycosylated VCAM1 protein (Fig. 2). These data provide strong evidence that inhibition of translocation by CAM741

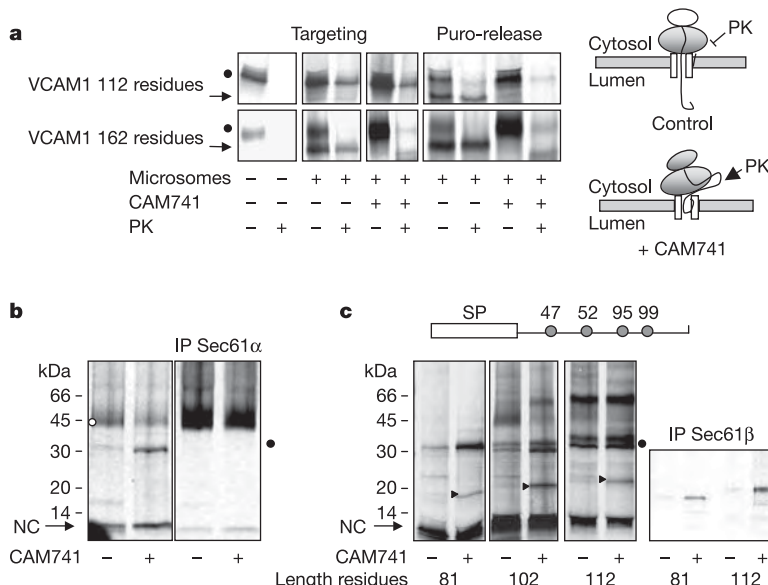


Figure 5 | CAM741 inhibits VCAM1 translocation at a post-targeting step and enhances interaction of the VCAM1 nascent chains with Sec61 β . **a**, *In vitro* targeting, translocation and proteinase K (PK) digestion of [35 S]methionine-labelled VCAM1 nascent chains (NCs). Arrow, NC with the SP cleaved off.

b, Targeted [35 S]methionine-labelled VCAM1 NCs of 92 amino-acid residues after chemical crosslinking with MBS (left) and immunoprecipitation (IP) of crosslinked NCs with a Sec61 α antiserum (right). Open circle, VCAM1 NC-Sec61 α crosslink; filled circle, NC. **c**, Targeted [35 S]methionine-labelled VCAM1 NCs after chemical crosslinking with BMH. Immunoprecipitations of crosslinked VCAM1 NCs were performed with a polyclonal antiserum against Sec61 β (right). Arrowhead, VCAM1 NC-Sec61 β crosslink. In **b** and **c**, the bands indicated by a filled circle are residual peptidyl-tRNA-NCs. A schematic representation of cysteine residues in the VCAM1 mature region is shown.

is also operative in living cells, as revealed by the cytosolic localization of VCAM1.

Taken together, our data indicate that CAM741 inhibits VCAM1 glycoprotein synthesis by specifically blocking its cotranslational translocation. First, CAM741 potently and specifically inhibits cell-free cotranslational translocation of the VCAM1 nascent chain across the ER membrane. Second, the VCAM1 SP is the principal determinant of cyclopetolide sensitivity. Third, translocation, but not targeting, is inhibited by CAM741 through a process involving the translocon component Sec61 β . Fourth, in the presence of CAM741 non-glycosylated, full-length precursor VCAM1 protein is synthesized but rapidly degraded through the proteasome degradation pathway in the cytosolic compartment of the cell. Last, this inhibitory process occurs in both cell-free and cellular systems.

We show here that SP-dependent blocking of the translocation process by a low-molecular-mass compound is possible without affecting the translocation of other proteins or compromising the translocation machinery. This offers therapeutic possibilities and can be exploited to identify substances that interfere specifically with the production of a secretory or membrane protein involved in disease.

METHODS

Reagents. CAM741 was dissolved in DMSO and stored at -20°C . For translocation experiments *in vitro*, CAM741 was added before the translation reaction and kept on ice for 30 min.

Plasmid constructions and RNA synthesis. VCAM1 cDNA was cloned from tumour necrosis factor- α -stimulated human umbilical vein endothelial cells by polymerase chain reaction (PCR) and cloned into pcDNA3.1 (Invitrogen). E-selectin and ICAM1 cDNAs were purchased from R&D Systems. Fusion constructs and truncated VCAM1 cDNAs lacking a stop codon were generated by PCR and subcloned into pcDNA3.1. The constructs encoding truncated VCAM1 NCs contained two C-terminal amino acids provided by the restriction site. Linearized plasmids were used as templates for creation of RNAs by the RiboMAX Large Scale RNA production System-T7 (Promega).

Translocation experiments *in vitro*. Translation/translocation assays with full-length cDNAs were performed *in vitro* with the coupled TNT reticulocyte lysate system and canine pancreatic microsomal membranes (Promega) in the presence of [^{35}S]methionine (Amersham) in a volume of 25 μl . Digestion with Proteinase K (12.5 $\mu\text{g ml}^{-1}$; Roche) was performed for 30 min on ice. For sedimentation of membranes, 20 μl of reaction mixture was diluted with 100 μl of 0.25 M sucrose, 20 mM EDTA, 50 mM triethanolamine pH 7.5 and layered on a 100- μl cushion of 0.5 M sucrose, 140 mM sodium acetate, 20 mM EDTA, 2.5 mM magnesium acetate, 50 mM triethanolamine, pH 7.5. Centrifugation was performed at 100,000 r.p.m. for 5 min in a Beckman TLA100 rotor. Pellets were dissolved directly in SDS sample buffer and supernatants were precipitated with two volumes of saturated ammonium sulphate solution. Translation, targeting and translocation assays *in vitro* with truncated VCAM1 NCs were performed with RNAs using rabbit reticulocyte lysate, canine pancreatic microsomal membranes and [^{35}S]methionine, in the presence of CAM741 (1 μM) or DMSO (vehicle control) in a volume of 25 μl . Targeting was performed for 30 min at 26°C . Release of the NCs from the ribosome was induced after 10 min of targeting at 26°C followed by treatment with 3.4 μl of 4 M potassium acetate, 2.6 μl of 20 mM magnesium acetate and 3 μl of 36 mM puromycin for 20 min at 26°C . The reaction mix was then diluted with 75 μl of 0.25 M sucrose, 150 mM potassium acetate, 40 mM HEPES pH 7.3, 5 mM magnesium acetate, 2 mM dithiothreitol and layered on a 100- μl cushion of 0.5 M sucrose, 500 mM potassium acetate, 40 mM HEPES pH 7.3, 2 mM magnesium acetate, 2 mM dithiothreitol. Centrifugation was performed in a Beckman TLA120 rotor at 50,000 r.p.m. for 4 min. Chemical crosslinking after sedimentation of membranes with targeted NCs was performed with 1 mM MBS (Pierce) or 1 mM BMH (Pierce) for 30 min at 25°C and stopped by the addition of 5 mM 2-mercaptoethanol and 100 mM Tris-HCl pH 7.5, followed by release of the NCs by the addition of 3 μl of puromycin and 2 μl of 4 M potassium acetate for 10 min at 30°C . Proteins were then precipitated with 15% trichloroacetic acid, followed by a wash with ice-cold acetone. Dried pellets were dissolved in SDS sample buffer. For immunoprecipitations after chemical crosslinking,

membranes were treated with 1% SDS in 50 mM Tris-HCl pH 7.5 for 5 min at 95°C , before the addition of a tenfold volume of denaturing immunoprecipitation buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.5, 0.2% SDS, 1% Triton X-100, 0.4 mM PMSF). Immunoprecipitations were then performed with a polyclonal antiserum against Sec61 α (Upstate) or a polyclonal antiserum raised against the peptide PGTPSGTNC of Sec61 β . Proteins were separated on ExcelGel SDS 8–18% or 12–14% polyacrylamide gels (Amersham), and dried gels were exposed to X-ray films.

Cellular assays. HEK-293 and COS-7 cells were cultured in DMEM medium supplemented with 10% FCS. Transient transfections were performed with Lipofectamine reagent (Invitrogen) for COS-7 cells or Superfect (Qiagen) for HEK-293 cells, in accordance with the manufacturer's instructions, and treated with increasing concentrations of CAM741. Western blot analysis of cell lysates was performed in accordance with standard protocols. A goat polyclonal anti-VCAM1, a mouse monoclonal anti-E-selectin (both from R&D Systems) and a mouse monoclonal antibody against β -actin (Chemicon) were used for detection. For proteasome inhibition, transiently transfected HEK-293 cells were incubated without or with 100 nM CAM741 in the presence of 5 μM lactacystin (Calbiochem) for 24 h before lysis and western blot analysis. Deglycosylation was performed with N-glycosidase F (Roche).

Received 31 January; accepted 18 April 2005.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements We thank A. Rot for generation of the Sec61 β antiserum, H. Jaksche for its purification, and B. Kappel, H. Pertl, L. Hofer and N. Lettner for technical assistance.

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LETTERS

A role for cell-cycle-regulated histone H3 lysine 56 acetylation in the DNA damage response

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DNA breaks are extremely harmful lesions that need to be repaired efficiently throughout the genome. However, the packaging of DNA into nucleosomes is a significant barrier to DNA repair, and the mechanisms of repair in the context of chromatin are poorly understood¹. Here we show that lysine 56 (K56) acetylation is an abundant modification of newly synthesized histone H3 molecules that are incorporated into chromosomes during S phase. Defects in the acetylation of K56 in histone H3 result in sensitivity to genotoxic agents that cause DNA strand breaks during replication. In the absence of DNA damage, the acetylation of histone H3 K56 largely disappears in G2. In contrast, cells with DNA breaks maintain high levels of acetylation, and the persistence of the modification is dependent on DNA damage checkpoint proteins. We suggest that the acetylation of histone H3 K56 creates a favourable chromatin environment for DNA repair and that a key component of the DNA damage response is to preserve this acetylation.

To uncover novel post-translational modifications, histone H3 was purified from *Saccharomyces cerevisiae* (Fig. 1a) and analysed by tandem mass spectrometry. From a digest with endoproteinase Arg-C, we identified two doubly charged parent ions with mass-to-charge ratios (m/z) of 617.83 and 638.85 that were derived from histone H3 54-FQKSTELLIR-63 (Supplementary Fig. S1). Fragmentation of the parent ions confirmed their identity as unmodified ($m/z = 617.83$) and K56-acetylated ($m/z = 638.85$) peptides. The fragmentation spectrum of the $m/z = 638.85$ parent ion revealed a mass difference between the y7 and y8 fragment ions (170.0983 Da) very close to that predicted for K56 acetylation (170.1015 Da) and significantly different from that expected for trimethyl lysine (170.1419 Da; Fig. 1b). The mass difference between the b2 and b3 fragment ions (170.1057 Da) was almost identical to that calculated for acetyl-lysine (170.1015 Da, Fig. 1b). K56 trimethylation was incompatible with the late retention time of the m/z 638.85 parent ion during reverse-phase liquid chromatography. Unlike acetylation, trimethylation preserved the positive charge of K56. Hence, a K56-trimethylated synthetic peptide eluted close to the unmodified peptide and much earlier than the K56-acetylated peptide during reverse-phase chromatography (Supplementary Fig. S1). We generated rabbit antibodies that recognized histone H3 acetylated at K56. In wild-type cells, these antibodies detected a single polypeptide that was absent from cells in which histone H3 K56 was mutated to arginine or in H3 purified from *Escherichia coli* (Fig. 1c). In addition, an excess of unacetylated K56 peptide over the antibody did not block the signal observed in wild-type cells (Fig. 1d). Subsequent western blots were performed in the presence of excess unacetylated peptide to ensure that detection was specific for K56 acetylation.

K56 acetylation occurred during passage through S phase and disappeared abruptly in G2 (Fig. 1e and Supplementary Fig. S2a).

Using acetylation with deuterated acetic anhydride to render the unmodified and K56-acetylated forms of histone H3 chemically equivalent, we estimated that as much as 20% of H3 was acetylated at K56 in asynchronous cells (Supplementary Fig. S3). Given that K56 acetylation was restricted to S phase, this implied that a large fraction of H3 was acetylated at K56. Before their incorporation into chromatin, newly synthesized histones are acetylated at multiple lysine residues and form a complex with chromatin assembly factor 1 (CAF-1)²⁻⁵. We found that H3 associated with CAF-1 was acetylated at K56 (Fig. 1g). In addition, newly synthesized H3 expressed from a galactose-inducible promoter was also acetylated in G1 cells, even though the majority of overexpressed H3 cannot be packaged into chromatin in these cells (Supplementary Fig. S2b). To provide further evidence that K56 acetylation occurred in newly synthesized histones, a strain in which histone H3 was expressed from a galactose-regulated promoter was released from G1 into the cell cycle. When the synthesis of new H3 molecules was inhibited by glucose, K56 acetylation was undetectable even though the cells completed one round of DNA replication (Fig. 1f, glucose). In contrast, when new histone H3 synthesis was induced with galactose, K56 acetylation occurred transiently during passage through S phase for at least two rounds of DNA replication (Fig. 1f, galactose, 1 and 3 h time points). This experiment showed that histone H3 K56 acetylation occurred predominantly in newly synthesized, rather than pre-existing, H3 molecules. Because K56 acetylation was transient, it was possible that the acetylation was removed either before or immediately after H3 deposition behind the replication fork. To address this, yeast extracts were fractionated by centrifugation to separate newly synthesized histones in the soluble fraction from histones packaged into chromatin⁶. When asynchronous cells were fractionated, we found that soluble histone H3 was below the level of detection and that most of the H3 acetylated at K56 was in the chromatin fraction (Fig. 1h). Thus, K56-acetylated histone H3 was deposited into chromatin during replication.

The biological function of K56 acetylation was assessed with yeast strains carrying mutations of K56 into alanine (K56A), glutamine (K56Q) or arginine (K56R). These strains did not have any pronounced defect in cell cycle progression but were sensitive to several genotoxic agents that, either directly or indirectly, result in DNA breaks during replication (Fig. 2a and Supplementary Fig. S4). The K56Q mutation, which mimicked constitutive acetylation, conferred slightly less sensitivity to DNA damage than other K56 mutations. The K56 mutants were not sensitive to ultraviolet or ionizing radiation (Fig. 2a and Supplementary Fig. S8c), and ionizing-radiation-induced histone H2A phosphorylation and H4 lysine 8 acetylation occurred normally in cells lacking histone H3 K56 acetylation (Supplementary Fig. S8). Thus, cells clearly have mechanisms for modulating chromatin structure in response to DNA damage that are independent of histone H3 K56 acetylation.

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Two other modifications of core histones have been implicated in the DNA damage response. Phosphorylation of a single serine residue (S129) near the carboxy terminus of histone H2A (H2A-X in higher eukaryotes) is rapidly induced in nucleosomes surrounding DNA double-strand breaks (DSBs) and is one of the best known markers of DSBs⁷⁻⁹. In addition, a mutation that compromises the four known sites of acetylation in the amino-terminal tail of histone H4 (*hhf1-10*) also results in sensitivity to DNA damage¹⁰. These four acetylation sites are redundant and the presence of a single intact lysine residue in the H4 tail is sufficient to protect cells against DNA damage¹⁰. The K56R mutants were markedly more sensitive than *hta* S129A or *hhf1-10* cells to camptothecin (CPT) (Fig. 2b). The *hhf1-10* and K56R mutants were sensitive to low doses of bleomycin at which the *hta* S129A mutant and wild-type cells were equally resistant (Fig. 2b). Thus, the modification of histone H3 K56 has a unique function in the response to DNA strand breaks that cannot be replaced by acetylation of the H4 N-terminal tail and has a far greater impact

on sensitivity to CPT and to bleomycin than H2A phosphorylation. The CPT sensitivity of the K56R mutant strain was partly suppressed by the expression of wild-type histone H3 from a high-copy plasmid (Fig. 2c). Conversely, expression of the K56R mutant from the same plasmid impaired the ability of wild-type cells to grow in the presence of CPT (Fig. 2c). Thus, sensitivity to CPT was modulated by the fraction of histone H3 molecules acetylated at K56.

Cells with the K56R mutation delayed mitosis in response to replication arrest and CPT-induced breaks (Supplementary Fig. S5 and data not shown). The G2/M checkpoint was therefore unaffected by the absence of histone H3 K56 acetylation. The acute sensitivity of K56R mutant cells to DNA damage was not a reflection of defects in non-homologous DNA end-joining (NHEJ; Supplementary Fig. S6). Because homologous recombination (HR) is a more faithful mechanism to repair DSBs than NHEJ, HR is the preferred repair pathway from S phase to G2, when K56 acetylation is most abundant¹¹. CPT is a cancer chemotherapeutic agent that causes single-strand breaks by

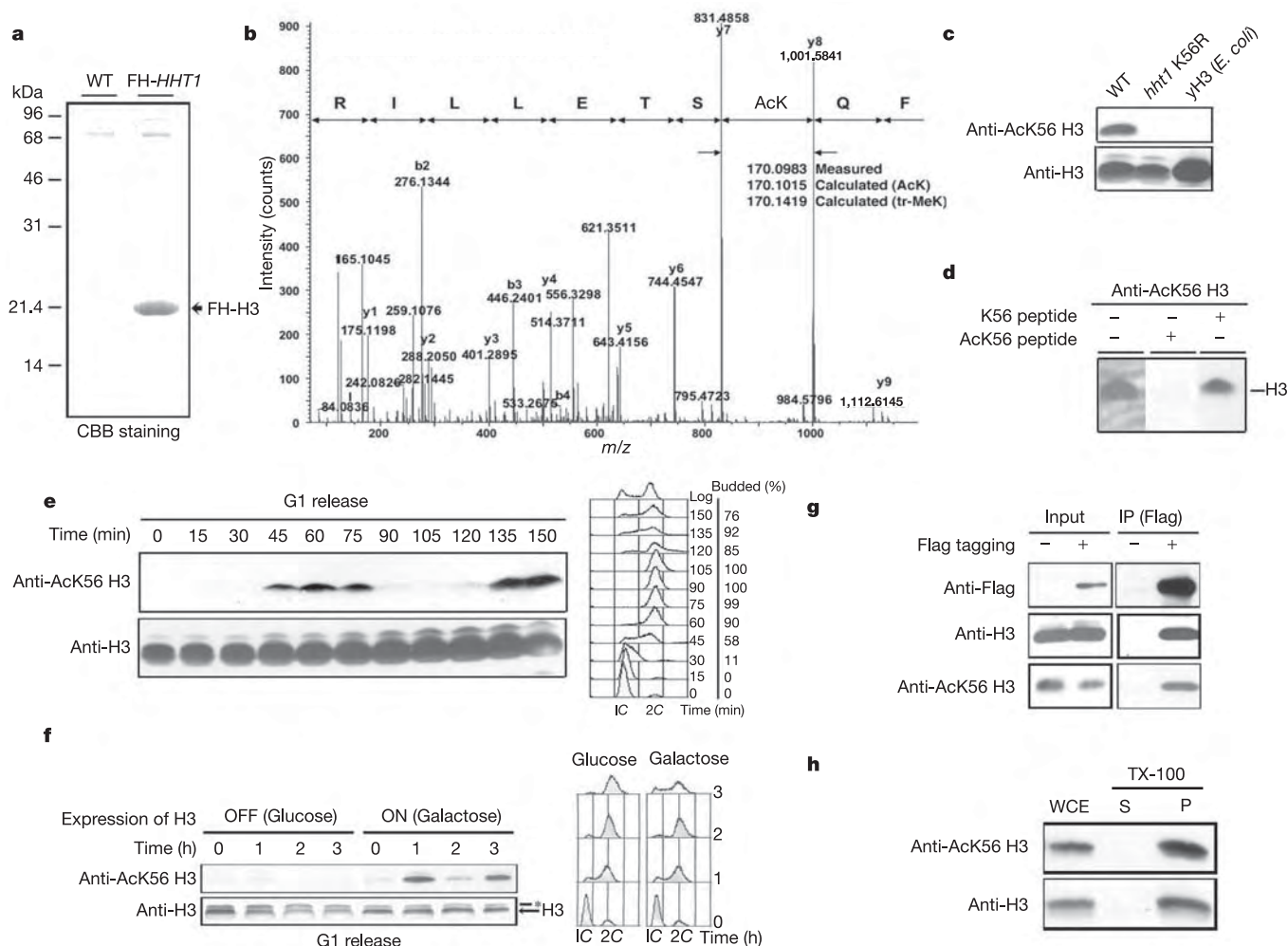


Figure 1 | Newly synthesized histone H3 is acetylated at K56. **a**, Flag-His10-H3 (FH-H3) purified from *S. cerevisiae*. WT, wild-type cells lacking the tag. FH-HHT1, cells expressing FH-H3. **b**, Collision-induced fragmentation of the parent ion (histone H3 54-FQacKSTELLIR-63) with $m/z = 638.85$. The arrows bracket the peaks that define the presence of acetyl-lysine (AcK). **c**, Western blots of wild-type and histone H3 K56R mutant cells (*hht1* K56R) were probed with antibodies against histone H3 acetylated at K56 (AcK56) or total H3. **d**, Whole-cell extracts probed with AcK56 antibody and either unacetylated or K56-acetylated H3 peptide as competitors (1,000-fold molar excess over the antibody). **e**, Wild-type cells were released from G1 into the cell cycle in YPD medium at 25 °C and histone H3 K56 acetylation was monitored at 15-min intervals. **f**, Cells in which the

only source of histone H3 was expressed from a galactose-regulated promoter (*hht1Δ hht2Δ P_{GALI}-HHT1*) were released from G1 arrest into fresh medium containing glucose or galactose to either repress or allow the synthesis of new histone H3. **g**, Cells were released from G1 and arrested just before initiation of DNA replication with a *cdc7* mutation. Cac1-3Flag was immunoprecipitated and bound histones were detected by western blotting. Input: 1.5% of input material. IP, immunoprecipitation. **h**, K56-acetylated histone H3 is present in the chromatin fraction. Spheroplasts prepared from wild-type cells in exponential phase were lysed in buffer containing 1% Triton X-100 (TX-100). After centrifugation, K56 acetylation was monitored by western blotting of the whole-cell extract (WCE), the soluble protein fraction (S) and the chromatin pellet (P).

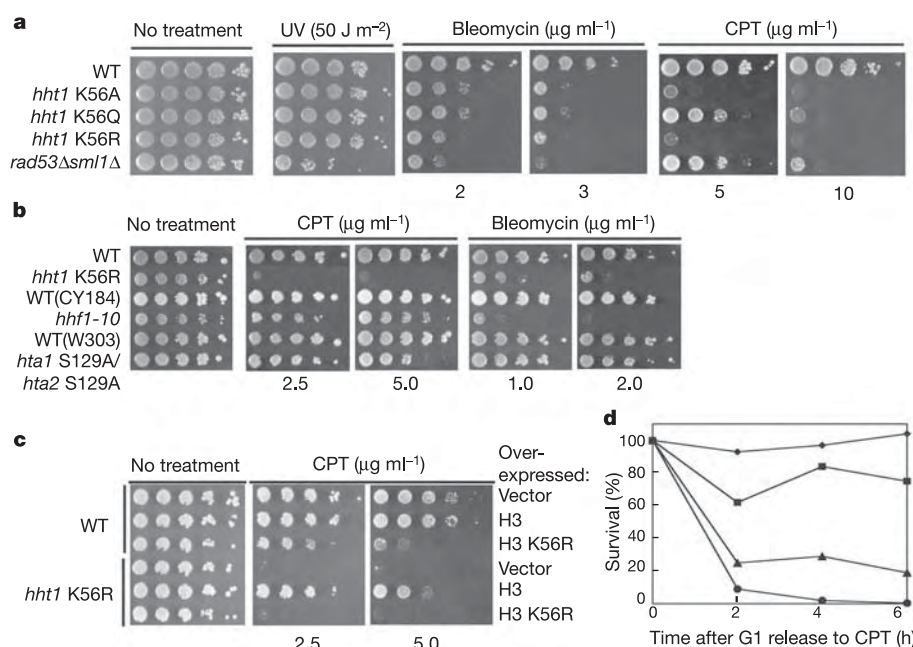


Figure 2 | Histone H3 K56 contributes to survival of camptothecin (CPT) and bleomycin-induced DNA breaks. **a–c**, Tenfold serial dilutions of each strain were analysed in plates containing either bleomycin or CPT. One set was exposed to ultraviolet radiation (UV). WT, wild type. **a**, Histone H3 K56 mutations confer bleomycin and CPT sensitivity. **b**, Histone H3 K56 is not redundant with the acetylation of the four N-terminal lysines of histone H4 (*hht1-10* mutant) or the phosphorylation of histone H2A (*hta1* S129A *hta2* S129A mutant). **c**, CPT sensitivity is determined by the abundance of the

histone H3 K56R mutant. Wild-type histone H3 or the K56R mutant were overexpressed from high-copy plasmids. **d**, Histone H3 K56 contributes to CPT survival in a Rad52-independent manner. The indicated strains were synchronized in G1 and released into the cell cycle in the presence of 10 μg ml⁻¹ CPT. The fraction of viable cells was determined as a function of time. Diamonds, wild type; squares, *rad52Δ*; triangles, *hht1* K56R; circles, *hht1* K56R *rad52Δ*.

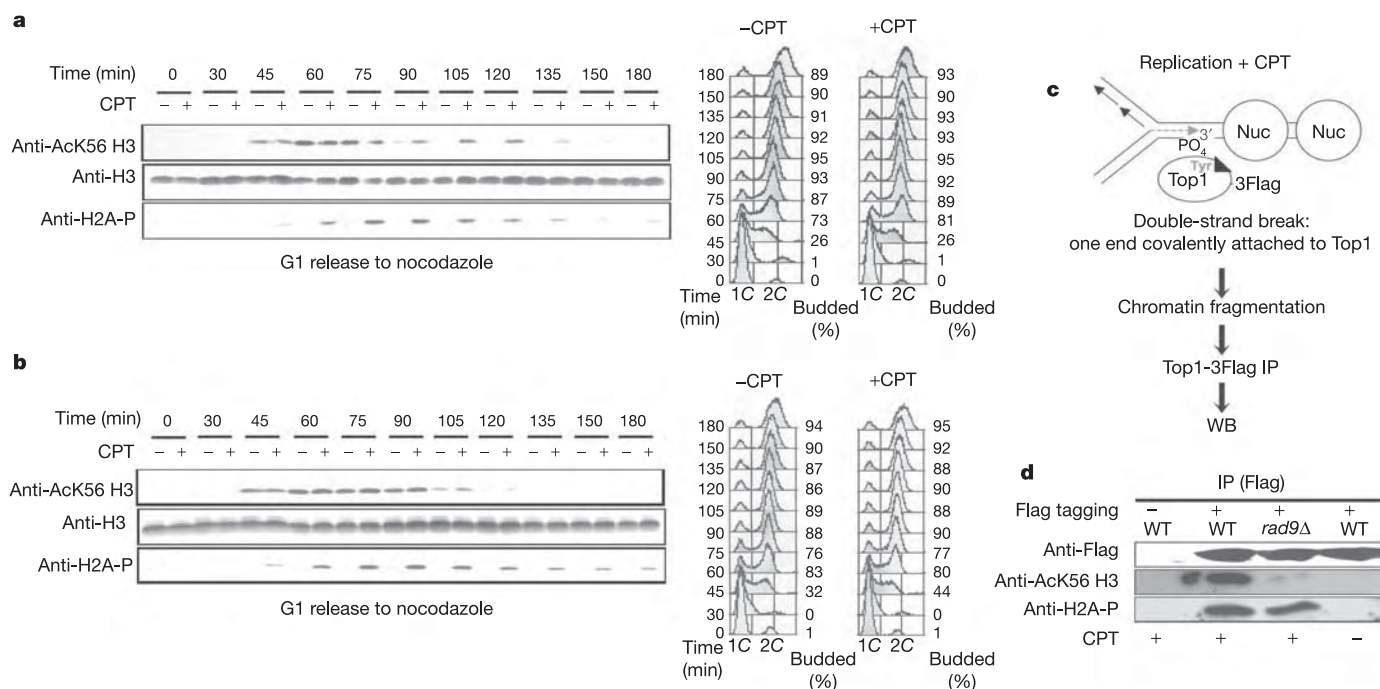


Figure 3 | Rad9-dependent retention of histone H3 K56 acetylation at sites of CPT-induced DNA breaks. **a, b**, Wild-type (**a**) and *rad9Δ* (**b**) cells were released from G1 arrest in the absence or presence of 10 μg ml⁻¹ CPT at 25 °C. Western blots of whole-cell extracts were probed for K56 acetylation, total histone H3 or H2A phosphorylation. **c**, Strategy for affinity purification of oligonucleosomes covalently attached to Top1 at sites of CPT-induced DNA strand breaks. During replication, CPT results in the formation of a covalent bond between a catalytic site tyrosine in Top1 and a DNA 3' -phosphate¹². The nick is then converted into a DSB through leading

strand replication¹². After chromatin shearing by sonication, oligonucleosomes derived from one side of the break were affinity-purified with antibodies against epitope-tagged Top1. Nuc, nucleosome; IP, immunoprecipitation; WB, western blot. **d**, Wild-type and *rad9Δ* cells expressing Top1-3Flag were released from G1 arrest for 50 min at 30 °C in 10 μg ml⁻¹ CPT to create DNA strand breaks covalently attached to Top1-3Flag. Chromatin fragments were affinity-purified with anti-Flag antibodies, and Top1-3Flag, K56-acetylated H3 and phosphorylated H2A were detected by western blotting.

inhibiting the release of DNA topoisomerase 1 (Top1) from DNA^{12,13}. These lesions are toxic mainly to S-phase cells, in which they are converted into DSBs during replication^{13,14}. Cells defective in HR, such as *rad52* mutants, lost viability during replication in the presence of CPT (Fig. 2d). However, K56R cells showed a more pronounced loss of viability than *rad52* mutants, and double-mutant cells were even more sensitive than K56R single mutants (Fig. 2d). The same was true for methyl methane sulphonate (Supplementary Fig. S7), an alkylating agent that is predominantly cytotoxic during S phase¹⁵. These results indicate that, in response to genotoxic agents that interfere with replication, histone H3 K56 contributes to cell survival through a Rad52-independent pathway. However, the results do not preclude a role for K56 acetylation in HR.

Because histone H3 K56 was crucial for survival from CPT-induced DSBs, we reasoned that cells might have evolved a mechanism to maintain high levels of K56 acetylation in response to DSBs. To address this, we monitored K56 acetylation during cell cycle progression. In the absence of CPT, K56 acetylation occurred transiently during passage through S phase (45–75 min, Fig. 3a). In cells exposed to CPT, K56 acetylation was detectable for much longer in G2 (up to 135 min; Fig. 3a). Even during chronic exposure to CPT, H3 K56 acetylation and H2A phosphorylation eventually disappeared with similar kinetics in G2 (Fig. 3a). Because H2A phosphorylation is an excellent marker of DNA damage^{7–9}, this indicated that deacetylation of histone H3 K56 was delayed until DNA repair was complete. Consistent with this, H2A phosphorylation and H3 K56 acetylation remained in *mre11* mutant cells in which the repair of CPT lesions was severely impaired (Supplementary Fig. S9a). In *S. cerevisiae*, DSBs induced by CPT during replication do not elicit a checkpoint within S phase, but the breaks eventually activate a delay in G2 phase⁸. Yeast cells lacking the DNA-damage checkpoint proteins Mec1 and Rad9 are sensitive to CPT⁸. The persistence of K56 acetylation in response to CPT was markedly reduced in *rad9* and *mec1* mutant cells (Fig. 3b and Supplementary Fig. S9b), even though the repair of CPT lesions was impaired, as revealed by the prolonged phosphorylation of H2A in G2 (Fig. 3b). To provide evidence that histone H3 K56 acetylation was retained at sites of DNA damage, we devised a strategy for the affinity purification of oligonucleosomes adjacent to DNA breaks. CPT triggers the formation of covalent adducts between Top1 and DNA^{12,13}. Oligonucleosomes covalently attached to epitope-tagged Top1 were affinity-purified with anti-Flag antibodies (Fig. 3c). In wild-type cells these oligonucleosomes contained both K56-acetylated histone H3 and phosphorylated H2A (Fig. 3d). The detection of these modifications was dependent on both the Flag epitope and the addition of CPT (Fig. 3d), showing that the purified nucleosomes were derived from genuine sites of covalent attachment of Top1 to DNA. Interestingly, nucleosomes purified from *rad9* mutant cells retained their H2A phosphorylation but had significantly smaller amounts of H3 K56 acetylation (Fig. 3d). These results suggest that DNA damage checkpoint proteins promote the repair of DNA breaks induced by CPT by delaying the deacetylation of histone H3 K56 that normally occurs during G2 phase in the absence of DNA damage.

Unlike other sites of acetylation, K56 is not within the N-terminal tail of H3. K56 is the last residue of the α N-helix, which is found only in histone H3 and precedes the histone-fold domain (Fig. 4a). The K56 ϵ -amino groups of each H3 molecule make water-mediated contacts with the DNA phosphodiester backbone at the entry and exit points from the nucleosome core particle¹⁶ (Fig. 4a). To assess the role of the K56 positive charge, we compared the chromatin structures of K56 mutant strains. In plasmid DNA, each nucleosome protects roughly one negative superhelical turn against the action of DNA topoisomerases¹⁷. Disruption of histone–DNA interactions decreases the negative supercoiling of plasmid DNA^{7,18}. The K56Q mutation that mimicked constitutive acetylation decreased the supercoiling of an endogenous plasmid compared with the K56R mutation or wild-type H3 (Fig. 4b). Consistent with loss of

histone–DNA interactions at the entry and exit points, nucleosomes from K56Q mutant cells were extensively digested by micrococcal nuclease, an enzyme that trims the inter-nucleosomal DNA and normally shows a strong pause at the border of the nucleosome (Fig. 4c). In contrast, the ends of nucleosomal DNA were less readily digested by micrococcal nuclease in wild-type and K56R mutant cells. The similarity between wild-type and K56R mutant cells in both assays (Fig. 4b, c) probably reflects the fact that about 80% of H3 is not K56-acetylated in asynchronous cells (Supplementary Fig. S3). These results indicate that histone H3 K56 acetylation weakens histone–DNA interactions at the entry and exit points in the nucleosome. Because other residues in the α N helix also contact DNA¹⁶, it seems likely that K56 acetylation exerts its function through enzymes that cause further structural alterations to nucleosomes.

A single persistent DSB is sufficient to promote chromosome mis-segregation and loss of cell viability. For this reason, DSBs caused by spontaneous collapse of the DNA replication fork or by genotoxic

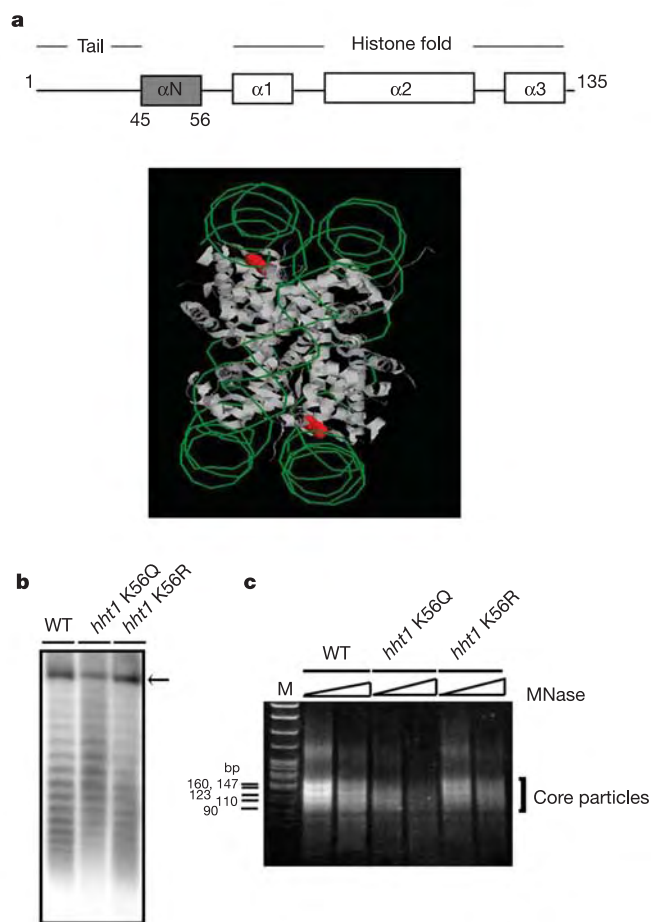


Figure 4 | The positive charge of histone H3 K56 is important for histone–DNA interactions at the entry and exit points from the nucleosome core particle. **a**, Top: the α N helix is located between the N-terminal tail and the histone-fold domain of histone H3. Bottom: the K56 ϵ -amino groups of the two histone H3 molecules (shown in red) make water-mediated contacts with the DNA (green) at the entry and exit points of the nucleosome core particle. **b**, DNA was isolated from wild-type (WT), K56Q and K56R mutant cells, and resolved through an agarose gel containing chloroquine. The superhelical density of an endogenous 2 μ plasmid was analysed by Southern blotting and hybridization to a DNA probe derived from the 2 μ origin. The nicked circular plasmid is indicated by an arrow. **c**, Nuclei were isolated from wild-type, K56Q and K56R mutant strains, and incubated with micrococcal nuclease (MNase). The DNA was purified, resolved in an 8% polyacrylamide gel and stained with ethidium bromide. Lane M, size markers.

agents need to be repaired throughout the genome. The fact that newly synthesized histones are acetylated at multiple residues^{2–5} and rapidly deposited behind the replication fork¹⁹ may create a chromatin environment that is favourable for the repair of DSBs irrespective of their chromosomal location. Because deposition-related acetylation is present before damage occurs, it potentially eliminates the need to recruit histone acetyltransferase enzymes to sites of DSBs. Therefore, the active recruitment of histone acetyltransferase(s) to DSBs, which has been reported for subunits of the NuA4/Esa1 complex^{10,20}, might be more crucial in G1 or G2 cells that do not have genome-wide acetylation of newly synthesized histones.

METHODS

Mass spectrometry. Flag-His10-H3 was resolved on an SDS–15% polyacrylamide gel and digested with endoproteinase Arg-C. The digest was analysed on a nano-LC pepmap reverse-phase chromatography C₁₈ column (75 µm × 150 mm; LC-Packings) connected to a Q star tandem mass spectrometer (Applied Biosystems/MDS Sciex). The mass spectrometer was externally calibrated shortly before the run, with the use of a standard peptide in MS/MS mode. This calibration results in a mass accuracy of about 10 p.p.m. in the range from about 70 to 1,000 Da, which is typical for most fragment ions.

Western blotting. Whole-cell extracts were prepared for SDS–polyacrylamide-gel electrophoresis with the use of an alkaline method²¹. Proteins from 2.5 × 10⁶ cells were resolved in SDS–15% polyacrylamide gels and transferred to nitrocellulose. The blots were probed with polyclonal antibodies against the C terminus of histone H3 (ref. 6), K56-acetylated H3, K8-acetylated histone H4 (Upstate Biotechnology) or the M2 mouse monoclonal antibody against the Flag epitope (Sigma), followed by horseradish-peroxidase-conjugated antibodies against rabbit or mouse IgGs (Amersham) and chemiluminescence detection (Pierce). Rabbit polyclonal antibodies were raised against a synthetic peptide containing acetylated K56 (H3acK56; RRFQacKSTELLIRKC, where 'ac' represents acetylation). The antibodies were isolated from the crude serum with two sequential affinity-purification steps with synthetic peptides cross-linked to SulfoLink (Pierce). Antibodies were eluted from the H3acK56 peptide column with 0.1 M glycine-HCl pH 2.5, neutralized with Tris base, and dialysed against Tris-buffered saline (TBS). Antibodies that recognized the unmodified peptide were removed by extensive immunodepletion over a column of non-acetylated K56 peptide. As an additional precaution, the resulting antibodies were used in western blots at 0.22 µg ml^{–1} in TBS in the presence of a 1,000-fold molar excess of non-acetylated peptide. Histone H2A phosphorylation was detected as described previously⁸.

Yeast strains and detailed methods. Details of yeast strains and other methods are provided in the Supplementary Information.

Received 4 February 2004; accepted 5 May 2005.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements We thank W. Bonner, M. Christman, J. Diffley, L. Drury, P. Fitzjohn, H. Nash, A. Kristjuhan, D. Lyon, C. Redon, D. Stillman, J. Sveistrup and S. Tanaka for reagents, and A. Castillo and A. Gunjan for critical reading of the manuscript. This work was funded by Cancer Research UK and the International Association for Cancer Research. H.M. was supported by postdoctoral fellowships from the NAITO Foundation and Cancer Research UK.

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naturejobs

Reality check

Sifting through feedback detailing the work experiences of *Naturejobs* readers, I was reminded of the chorus to a relatively obscure Lou Reed song that goes: "What's good? Not much at all". Most correspondents were complaining about poor supervision for students and postdocs, noting that the broadly positive sentiments expressed by the writers of our Graduate Journals, and in other articles, failed to resonate with them.

One writer said that there are "many more graduate-student horror stories for every rare and unusual experience" reported in our Graduate Journals. Another wrote that his experience failed to square with what he called our "rose-coloured" view, adding that "erratic supervision and an experimental design that has only recently revealed its considerable weaknesses have left my fascination with science exhausted". Still another said that many institutions and supervisors are chronically lacking at providing postgrads with guidance, and accuses *Naturejobs* of saying students should just grin and bear it, and "help each other out to get through it".

The level of vitriol in some of these responses tells me

that these people are frustrated by the lack of control that they have over their destinies. But although I agree with punk-rock legend John Lydon that "Anger is an energy", I'd add that these disgruntled readers should also listen to the Eric Clapton refrain: "It's in the way that you use it".

On that note, another writer pointed out that rather than complaining or expecting organizations to bail you out, you should be "proactive by taking steps to improve your own situation".

That sentiment has been the goal of *Naturejobs* from the start. We aim to give solutions and offer advice rather than wallowing in a culture of complaint. In essence, we're heeding the words of English rapper The Streets and focusing on "just trying to stay positive".



Paul Smaglik, *Naturejobs* editor

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MOVERS

Harry Stylli, chief executive and president, Sequenom, San Diego, California



2004–05: President and chief executive, Xencor, Monrovia, California

2002–03: President and chief executive, CovX Pharmaceuticals, San Diego

1995–2001: Senior vice-president, Aurora, San Diego

1985–95: Drug-discovery research manager, Glaxo, London

Harry Stylli's hobby, cryptozoology, speaks volumes about his career choice. Cryptozoology is the search for and study of animals whose existence is disputed. In some ways, it is not much different from his career in drug discovery, where he looks for therapies that don't yet exist.

Stylli began his career by combining science with industry: he worked on his PhD in pharmacological chemistry at King's College London with the help of a grant from Amersham International, a diagnostic imaging company now part of GE Healthcare.

His next stop was at industry giant Glaxo (now GlaxoSmithKline) where the self-described "frustrated entrepreneur" did something considered unusual at the time: he completed an MBA degree through the Open University. The degree allowed him to combine his understanding of science with the knowledge of the demands of business. He applied these lessons to his job, integrating elements of manufacturing strategy to the drug-discovery process.

After almost ten years of working in big pharma, in 1995 Stylli left to pursue his entrepreneurial bent in San Diego, California. There, he co-founded Aurora Biosciences. Taking a chance at Aurora was an invaluable experience that helped define his career, explains Stylli. It allowed him to go through an entire life cycle of a company, from founding to building and eventually leaving.

In 2001, the company was sold to Vertex Pharmaceuticals of Cambridge, Massachusetts, providing an objective measure of success for the firm and making Stylli a healthy profit. He is proud that the technology and people associated with Aurora early on remain leaders in the biotech industry. "Aurora 'DNA' is very much alive in other companies," he says.

Since then, Stylli has been at the helm of Xencor in Monrovia, California, and San Diego-based CovX. But he says his most significant challenge will be in his current job as chief executive and president of Sequenom, a San Diego genetic-analysis tool maker. "This is a company that has tremendous potential and promise, and hasn't managed to realize it yet," he says.

Stylli believes that biotechnology will be the engine of innovation in the forthcoming "age of healthcare". In this new age he predicts there will be dramatic changes in healthcare, with biotech bringing into development therapies that will move what was once viewed as science fiction or myth into reality. ■

NUTS & BOLTS

Change management

You've probably heard the saying "the only thing constant is change". When it comes to careers, that axiom certainly holds true. My career is no exception. After an interesting and rewarding year of writing in this space, my role is evolving, as is the column. In response to reader interest, the column is moving to an electronic medium, and we're adopting a Q&A format.

It seems only fitting, then, that I devote this column to adapting to change. From Charles Darwin to management guru Peter Drucker, writers have been fascinated by this topic, which is relevant to both research and workplace survival.

The questions I'll leave with you relate to three key skills for adapting to change and evolving in your career. Those skills are your ability to continuously develop, to establish a niche, and to let go of what is no longer useful or meaningful.

In response to changing influences, particularly in the continuously evolving world of science, the ability to keep developing takes on added significance. Ask yourself: "In what areas do I want to focus my own learning and development to continually recreate my career?"



With Deb Koen
Careers consultant

Establishing a niche to address emerging needs in science is a second strategy for career management. Survival in today's work world requires that you find a balance between differentiating and fitting in. Ask yourself: "How can I build on my unique qualities to fill a gap?"

Along with building on strengths, adapting to

change means you must say good-bye to the familiar and let go of that which is no longer useful or meaningful. With limited resources and changing agendas, distinguishing between what is essential or extraneous becomes a critical survival skill. Ask yourself: "Which core qualities, skills and responsibilities are integral to my work and identity, and which can I let go of?"

As your career evolves, please join me in my new setting and format (www.naturejobs.com). Here you'll have an opportunity to submit your own career management and job-search-related questions as well as review suggestions to questions posed by readers throughout the scientific community. ■

Deb Koen is acting president and chief executive of Career Development Services and a columnist for *The Wall Street Journal's CareerJournal.com*.

GRADUATE JOURNAL

The cost of moving abroad

I was chatting with a company manager in mid-career who was being transferred to Brazil for international training. I was amazed at how much his company helped him and his family in the move. This included renting a house, searching for a good school, paying the school fees, finding a job for his wife and arranging to get the family an extra car.

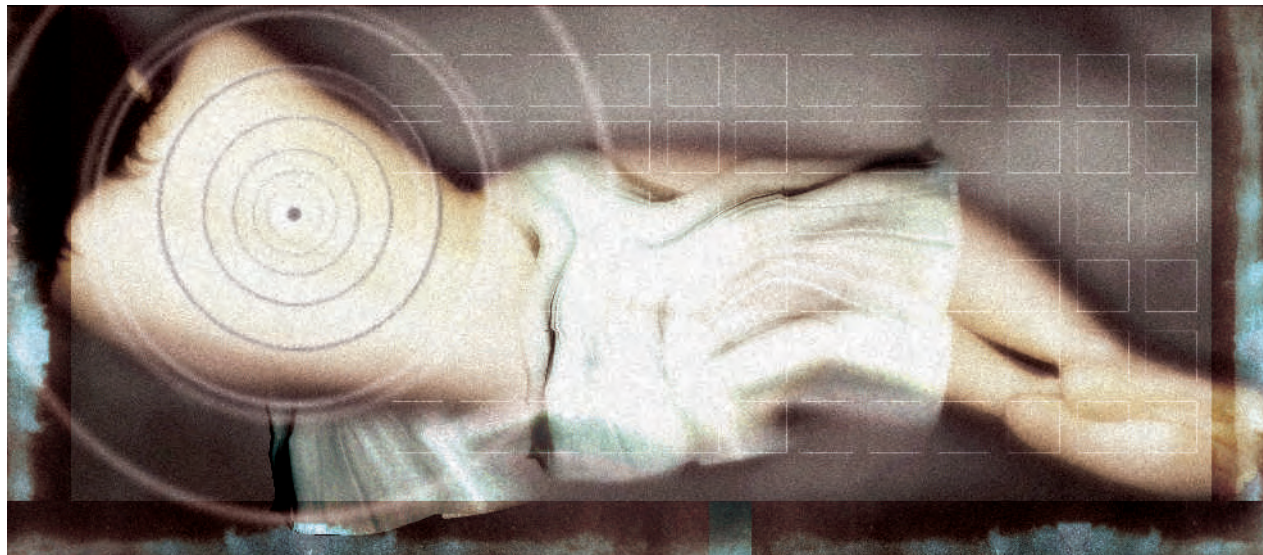
During our talk, I couldn't help drawing parallels with scientists. Many choose to pursue a PhD in a US lab, so they look for a suitable position and relocate. Should they be keen on a postdoctoral position in, say, Spain afterwards, they move again. And so on. Hence, the scenarios for those in business and science seem similar, except of course for how much help they're likely to get from an employer. Although some universities do their best to help find housing, a spouse's job and childcare, the level of help depends on the institution and country. Rarely does a university provide all the assistance of a large company.

Why do business managers require an economic incentive to move, whereas researchers are left to make do mostly on their own? Is it supply and demand? Are there many scientists but a dearth of qualified businesspeople to tap for international experience? I doubt it: the business world is just as competitive as that of science. But traditionally, each world emphasizes different rewards. Also, of course, public universities just don't have the money. ■

Tobias Langenhan is a first-year graduate student in neuroscience at the University of Oxford, UK.

The chemistry between us

Highs and lows.



JACEY

Laura Nelson

I sat on the bed as she lay sleeping. It was the last time.

"How could you not tell me?" I whispered fiercely. She was unaware of my presence; face down on the pillow, hair awry. I was furious with her silence, with her surreptitiousness, with her betrayal. I was furious that I couldn't, any more, be a slave to what she had become.

This girl had innate beauty, like an orchid. With the covers tossed aside, I saw her whole, naked body. Bitterly, I watched her back, flawless and pale. Her hair against her back was a sharp contrast of dark on white. Exquisite hair, naturally aubergine, straight as a taut wire, but soft as fur. I saw her neck underneath it. I'd always loved her neck: slim and tall.

My eyes scanned her neck and shoulders, and there they stopped with the sadness that now hit me, for I knew what was within that exquisite frame, under her skin, now part of her body. And not just her body, but her head too, because the metal box that was implanted in her chest governed the electrical stimulation of her brain, and dictated her moods. How could I have guessed that I would have to compete with an inanimate object — a mesh of wires, a robot — pulsing at regular intervals, triggering the release of natural brain chemicals into her pristine flesh? Was I the one making her happy, or was it her own chemistry, kick-started into action by the fastidious tweaking of a surgeon's fingers?

And it had made her happy, she said, since she had got the device a year ago, when we had both been 18. I remember the two of us, feeling as if we could conquer

the world, powerful in our new-found love. I needed nothing else. I was happy. But she — she was not — she needed the metal toy that had become as popular among our contemporaries as the latest computer games. Why, I asked her, as she sat combing my wet, unruly hair on a warm, fresh night by the ocean, when she had me: her girlfriend, her lover? She had felt lost, she replied. Like so many other people, she was complaining that the effects of antidepressant drugs weren't potent or continuous enough. After the implant, she didn't regret it. She felt much better, she said. And there it stayed, in her chest, pumping, through the day, through the night — her brain cells drowning in her own secretions, her temperament soaring. So she said.

Why then, did I begin to see things change for the worse? She became unpredictable, one moment ecstatic, the next moment down. There were no more care-free nights by the ocean. The girl I had known and loved seemed more distant, more distracted. When I broached the subject, daring to suggest the implant might be influencing her personality in ways she hadn't wished for, she scorned me with acerbic words.

Then we found out that other recipients of the device were experiencing similar and more sinister effects. The stories began to surface: hush-hush tales of friends who suddenly developed violent tempers, who turned on their partners and their families, and recoiled into states of despair. Then came rumours of suicides. Initially, reports of friends of friends floated back to us like thick black oil brought in by the tide. Now I had found out that one of our best friends

at school had stabbed herself, after the mechanical meddling had finally worked her weary brain to the limit.

She stirred and woke. I must have been frowning because she said: "What's wrong?"

I said: "Gabby killed herself."

"Yes," she said.

"Aren't you worried?"

"What?" she said.

"You might...do the same."

She sighed. "Oh, honeybunny." She paused, as if at my naivety.

Then she said: "I did...try. But I was rescued."

Seeing my dismay, she added: "Baby, don't worry, the pills have all gone. We don't have the pills in the house now. I won't be tempted."

I felt my anger swelling again. I stepped off the bed and stood up. My throat was plugged.

Sweat sprouted on my forehead. "You didn't tell me," I said. My voice was mellow and threatening.

She was crying now. Her body hunched up, her knees were bent into a fetal position; her hair was still sprawled and tears collected in pools above her lower lashes. I sat down on the bed, and gently took her ashen hand; it was hot and clutched. I prized open her fingers. The razor blades that she had been guarding fell like blossom on to the bed, winking at me menacingly in the low light. I laid her hand back down and stood up, trying to avoid her imploring, watery eyes — which would never try to look into my soul like that again — as I turned to walk out of the room. ■

Laura Nelson is a writer based in London.